

CAMPYLOBACTERS IN ENTERIC INFECTIONS OF CATTLE AND SHEEP

VOLUME I

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1984



TO MY WIFE

GRACIELA

AND MY SON

MARIANO



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DECLARATION

The work on campylobacters presented in this thesis was carried out by myself except for some collaborative experiments which are clearly indicated. A full role was played in the design of such multi-discipline work and analysis of the results. Some areas of the work reported in this thesis were part of a large and continuous project concerned with cause and control of neonatal calf diarrhoea, and consequently some of these results include data of other enteropathogens which have been obtained by my colleagues at the A.D.R.A., Moredun Research Institute.

Horacio Raúl Terzolo

July, 1984

## UNIVERSITY OF EDINBURGH

ABSTRACT OF THESIS <sup>7.9</sup> (Regulation 69)

of Candidate ..... Horacio Raúl Terzolo .....

.....

Degree ..... Doctor of Philosophy ..... Date ..... July 1984 .....

of Thesis ..... Campylobacters in enteric infections of cattle and sheep. .....

The primary aim of this study was to investigate the prevalence of campylobacters in cattle and sheep and their relationship with enteric diseases. Material obtained from surveys provided epidemiological information and strains to carry out taxonomical and pathological studies.

Campylobacters were isolated from 45.6 per cent of 693 diarrhoeic and 49.6 per cent of 125 non-diarrhoeic cattle and from 33.3 per cent of 24 diarrhoeic and 26.8 per cent of 127 non-diarrhoeic sheep. No significant relationship was found between the excretion of different Campylobacter spp. and diarrhoea.

In cattle C. jejuni was isolated from 22.1 per cent, C. coli from 6 per cent, "C.ointestinalis" (CHI) from 15.6 per cent and C. fetus from 3.4 per cent of 860 animals examined. Campylobacters were isolated from 88 per cent of the calf neonatal diarrhoea outbreaks investigated, having a higher isolation rate (45.1 per cent) than those of Cryptosporidium sp. (32.8 per cent), rotavirus (23.5 per cent), coronavirus (7.6 per cent) and K99+ E. coli (2.6 per cent). Rotavirus was significantly correlated with diarrhoea in most outbreaks, Cryptosporidium sp. in some and K99+ E. coli in a few. Campylobacters were isolated more frequently from calves infected with Cryptosporidium than from animals without a detectable excretion of oocysts. Fewer dairy calves younger than 1 month old excreted C. jejuni and C. coli than beef animals whilst the excretion rate of CHI and C. fetus and any of the other enteropathogens was similar. The means of improved bacteriological procedures 8 per cent of the calves were found to simultaneously excrete 2 or 3 Campylobacter spp.

In sheep C. jejuni was isolated from 14.6 per cent, C. coli from 6 per cent, "C. fecalis" from 8.6 per cent and C. sputorum subsp. bubulus from 2 per cent of 151 animals examined. Lambs may become naturally infected from the 28th day of life and excrete different Campylobacter spp. for short periods. No relationship was found between Campylobacter spp. excretion of dams and their corresponding nursing lambs.

In naturally infected calf faeces stored at +4°C campylobacters could survive for more than 3 months but the isolation rate obtained from 554 faeces significantly decreased from the 7th day of storage.

Colonial and microscopic morphology, coccal transformation and nalidixic acid (Nal), phenyl tetrazolium chloride and cephalothin tolerance were useful to differentiate 3 campylobacter groups which comprise 2 related species each: jejuni/coli GC (C. jejuni and C. coli), fetus GC (CHI and C. fetus) and "atypical" fetus GC ("C. fecalis" and C. sputorum subsp. bubulus). C. jejuni was differentiated from C. coli by hippurate test. C. fetus, CHI and "atypical" fetus GC by H<sub>2</sub>S production from FBP (Iron medium) and TSI. C. sputorum subsp. bubulus was differentiated from "C. fecalis" by catalase test. All bovine isolates were biochemically indistinguishable and 67.9 per cent serologically related to strains previously described as porcine CHI or bovine "C. fecalis". "C. fecalis" and C. sputorum subsp. bubulus were unique in becoming sensitive to Nal in agar containing Fe<sup>++</sup> or Fe<sup>+++</sup>. CHI strains produced black pigment in iron containing agar and a defect which was increased by exposure of plates to sunlight and inhibited by Nal. Capsules were detected in most campylobacters.

Single enteric infections were studied in gnotobiotic calves and lambs using C. jejuni strains in calves and lambs and C. coli and CHI in lambs. Campylobacters colonized and were maintained in high numbers in the large bowel but in the small intestine the number generally decreased with time. A mild disease was produced characterised by mucoid faeces and minor pathological alterations mainly restricted to the ileum and large bowel.

ACKNOWLEDGEMENTS

This work was carried out in the Department of Veterinary Pathology, Edinburgh University Field Station and was supported financially by a scholarship awarded by the "Instituto Nacional de Tecnología Agropecuaria" (INTA), Argentina.

I wish to extend my sincere thanks to Dr. G.H.K. Lawson, my supervisor, for his constant encouragement, advice and constructive criticism throughout this study.

I have pleasure in thanking the following people from the department: Miss P. Wooding and Mrs. N. Blair for their crucial and efficient support in the bacteriological laboratory; Mr. A.C. Rowland for his advice on pathological interpretation; Mr. N. MacIntyre, Mr. C. Nicolson and Mr. B. Kelly for their assistance in the pathology laboratory; Mr. D. Penman and Mr. C. MacFarlane who taught me the use of the electron microscope and prepared the photomicrographs; Dr. G. Scott for his invaluable statistical advice throughout; Mrs. H. London for her help with references; Mr. D. Laing, Mr. R. Beck, Dr. C. Munro and Mr. M. Slater for their help in the sampling of animals; Dr. J.L. Leaver for biochemical advice; and Mr. R. Munro for expert preparation of illustrations.

I am also very grateful to the Moredun Institute for the provision of animals and facilities and to the following members of staff for their advice and assistance in their relevant fields: Dr. D.R. Snodgrass, Mr. K.W. Angus, Miss I. Campbell, Dr. D. Sherwood and Mr. M. McLauchlan. Mr. B. Mitchell and Mr. C. McVittie and their staff for procuring and maintaining the gnotobiotic animals; and Mr. E.W. Gray and his staff for the electron microscopic photographs of cultures.

In addition, I would like to express my thanks to Mr. W. Deas who made the initial contact with the department. I express my gratitude



to all my friends in this country who gave support and encouragement in difficult times.

Finally, I would also like to express my deepest love to my wife Graciela, who not only gave me moral support and companionship but also typed this manuscript and prepared the graphs.

LIST OF ABBREVIATIONS

Ab	pyloric area of abomasum
A.D.R.A.	Animal Disease Research Association
AI	after incubation
AMP	adenosine monophosphate
ANA-O <sub>2</sub>	anaerobic atmosphere
ANA-O <sub>2</sub> -plus	strict anaerobic atmosphere
BAB	blood agar base
BABA	blood agar base plus agar
BSA	Brucella semisolid agar
C	cephalothin
Ca	caecum
CBA	Columbia blood agar
CBAA	CBA plus agar
CBA/TPB	biphasic slopes
CFU	colony forming units
CHI	<u>"Campylobacter hyointestinalis"</u>
CHO	Chinese hamster ovary
CLO's	campylobacter-like organisms
CNW	catalase negative or weak campylobacters
Co	colon
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetate
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
ETEC	enterotoxigenic <u>Escherichia coli</u>
FBP	iron-bisulphite-pyruvate supplement
<u>fetus</u> GC	<u>fetus</u> group campylobacters
HA	haemagglutination test
HEHA	haemadsorption-elution-haemagglutination assay
H <sub>2</sub>	hydrogen rich microaerophilic atmosphere
H <sub>2</sub> S-FBP	hydrogen sulphide from Iron medium
i/v	intravenous
<u>jejuni/coli</u> GC	<u>jejuni/coli</u> group campylobacters
K99 <sup>+</sup> EC	K99 <sup>+</sup> <u>Escherichia coli</u>
LO	large organisms
LT	heat-labile enterotoxin

MG	methyl-green
MG-DNase	methyl-green DNase agar
MIC	minimum inhibitory concentration
Minca-Is	Minca-IsoVitalex agar
MLN	mesenteric lymph nodes
N <sub>2</sub>	nitrogen rich microaerophilic atmosphere
Nal	nalidixic acid
Nal-inv	nalidixic acid resistance inversion
NARTC	nalidixic acid resistant thermophilic campylobacter ( <u>C. laridis</u> )
NAS	nutrient agar slopes
NB	nutrient broth
NB-FBP	nutrient broth with iron-bisulphite-pyruvate supplement
NBGT	novobiocin-brilliant green-trimethoprim agar
O <sub>2</sub>	aerobic atmosphere
P	Preston agar
P1	Preston agar without nystatin
P2	Preston agar without nystatin and with half the concentration of rifampicin
PAS	periodic acid Schiff
PBS	phosphate buffered saline
PHA	passive haemagglutination test
PI	post-inoculation
PIA	porcine intestinal adenomatosis
PN	Preston-nystatin agar
PN-CBA	Preston-nystatin-Columbia blood agar
Re	rectum
RNA	ribonucleic acid
RNBGT	rifampicin-novobiocin-brilliant green-trimethoprim agar
S	Skirrow's agar
(S)	standard deviation
SA	Skirrow's agar plus agar
SEM	scanning electron microscopy
SIM	sulphide-indol-motility medium
site 1	duodenum
site 2	jejunum
site 3	mid gut
site 4	lower ileum without Peyer's patches

site 5	lower ileum with Peyer's patches
SLM	Sabouraud liquid medium
SN	Skirrow's-nystatin agar
SPF	specific pathogen free
SO	small organisms
ST	heat-stable enterotoxin
TEM	transmission electron microscopy
TM	modified Brewer thioglycolate medium
TPB	tryptose phosphate broth
TSI	triple sugar iron agar
TTC	2,3,5-triphenyl-tetrazolium chloride
WHO	World Health Organization
w/v	weight/volume
Y-1	mouse adrenal tumour cells (Y-1)
YNB	yeast extract nutrient broth medium

CHAPTER 1Introduction

Diarrhoeic diseases are one of the major causes of neonatal mortality in animals. Calf neonatal diarrhoea is a complex disease that causes important economic losses all over the world. In Britain, the financial losses to farmers are difficult to estimate because calf mortality due to diarrhoea has not been accurately determined and there is an unrecorded cost of treatment and labour. Different surveys in Britain indicate that calf mortality varies between 24 per cent and 5.5 per cent in animals up to 6 months of age, with a mean mortality of 5 per cent for calves up to 3 months of age in rearing units as reviewed by Sherwood (1982). Despite that considerable progress has been achieved during recent years in improving our knowledge of some of the multiple infectious agents of calf neonatal diarrhoea this disease still remains largely unchecked (Tzipori, 1981). To-day, calf diarrhoea is still not completely understood, not only because of its complex aetiology but also because of the interaction of different infectious agents superimposed on environmental, nutritional, immunological and genetic factors. The relationship between a diversity of biochemical, physiological and microbiological processes that occur in the gut makes research into diarrhoeic diseases a very difficult subject. All major groups of microbes such as bacteria, protozoa, yeast and viruses - are present in the intestines and the development of microbial populations in the alimentary tract starts soon after birth and involves many host and microbial interactions. One of the objectives of research of recent years has been to identify pathogenic from

non-pathogenic microorganisms recovered from the intestinal environment in the laboratory. Many of the bacteria able to produce diarrhoeic diseases are also commensals of the intestinal tract - such as Escherichia coli or Clostridium spp. - and the distinction between saprophytic and enteropathogenic strains of the same species has been only achieved after many years of research work.

In the past, Escherichia coli was considered one of the major causes of calf neonatal diarrhoea but this concept has changed since the late 1960's when viral agents were found to be involved. Most of the Escherichia coli strains enteropathogenic for ruminants - calves and lambs - have the pilus-like protein structure K99 responsible for the attachment of the bacteria to the epithelial brush borders of the small intestine (jejunum and ileum). In general, such strains elaborate enterotoxins, in ruminants more frequently of the heat stable type (ST), and these strains are called enterotoxigenic E. coli or ETEC. ST specifically activates the intestinal fluid secretion and may also have a depressive effect on the muscularis. As a consequence of these actions fluid accumulates in the intestinal lumen with consequent diarrhoea. The ST induces severe or mild histological changes in newborn calves, characterized by fusion and stunting of the villi and replacement of enterocytes by immature cells. The K99 antigen can be serologically detected if the strains to be tested are grown in minimum medium that does not enhance the production of a K-polysaccharide antigen which could make ETEC K99<sup>+</sup> strains non-agglutinable. A more sensitive method for the detection of K99 antigen in faeces is the enzyme-

linked-immunosorbent assay (ELISA). Calves can be passively protected via colostrum if the mothers are vaccinated with a purified K99 antigen extract (Sherwood, 1982).

Since the discovery of rotaviruses in 1969 these are now considered one of the most frequent agents associated with field outbreaks of calf neonatal diarrhoea. Isolates from different animals are morphologically similar and share a common group antigen. By electron microscopy (EM) they are reo-virus-like of 65-70 nm in diameter and possess a double layered capsid. Immunofluorescent studies have shown that viral replication primarily occurs on the epithelial cells localized on the sides and on the tips of the villi of the middle and proximal small intestines. Histological examination of the affected intestinal areas reveals shortening of the villi, replacement of columnar epithelium with cuboidal immature cells and infiltration of mononuclear cells. The two most sensitive laboratory techniques for practical diagnosis of viral particles are the ELISA technique and the EM. Rotavirus are commonly observed in faeces of both diarrhoeic and apparently healthy calves and no variation in virulence of calf rotavirus isolates has been demonstrated. Vaccination of pregnant cows with inactivated rotavirus and of recently born calves with attenuated calf rotavirus has been attempted, but these vaccines are still under examination (Sherwood, 1982; Tzipori, 1981).

Coronavirus were first observed in 1972. They are a group of lipid containing pleomorphic, enveloped RNA viruses of 80 to 150 nm surrounded by a fringe of projections of two lengths, a characteristic feature of bovine coronavirus. A single bovine serotype specifically

causes diarrhoea in calves. Immunofluorescence studies have shown the virus in the epithelial cells throughout the small intestine and colon. Experimentally, the virus induces more severe clinical disease and intestinal damage than rotavirus in gnotobiotic calves. The significance of coronavirus as an enteropathogen has yet to be established in field surveys. An attenuated live vaccine has been developed and it was tested in combination with attenuated rotavirus, its efficiency under field conditions has to be proved (Sherwood, 1982; Tzipori, 1981).

Other viral agents - such as astrovirus, parvovirus, calicivirus, fringed particles, "Breda agent", "petit virus rond" (Lyon 1) and other viral particles provisionally called Lyon 3 and 5 - have been also detected by EM examination in association with diarrhoeic disease of neonatal calves but the role, significance and prevalence of these new viral particles have to be elucidated (Moussa, Dannacher and Fedida, 1983).

During the last years, calf Cryptosporidium sp. has also been shown to be a frequently occurring enteropathogen. Oocysts can be detected in Giemsa or modified Ziehl-Neelsen faecal smears and the parasite occurs generally in association with other agents but some outbreaks have been attributed solely to this protozoa. Field outbreaks of calf diarrhoea which involved only Cryptosporidium sp. were associated with moderate clinical symptoms and low mortality but in contrast diarrhoeic outbreaks in lambs were of higher mortality. Cryptosporidium sp. is distinguished from other enteric coccidia because it is an apparently extracellular parasite which adheres to the brush borders of the epithelial cells, the oocysts are small



(4  $\mu$ ) and it only affects new born animals. Inoculation of oocysts into calves induces diarrhoea and the organism is found adhering to brush borders of enterocytes throughout the small and large intestine but the terminal ileum is usually most severely infested. On the other hand, inoculated newborn lambs invariably die but infection at older than one week of age is seldom fatal. Antibiotics and other coccidicidal or coccidiostatic drugs are ineffective (Tzipori, 1981; Sherwood, 1982; Naciri and Yvoré, 1983).

Other specific diseases are sporadic and may occur in older animals. Salmonellosis is characterized by septicemia, sometimes accompanied by diarrhoea. The disease may reach epidemic proportions in calves which are commonly affected between 3 to 6 weeks of age. The two most frequent serotypes are Salmonella typhimurium which also infects many other animal species and, Salmonella dublin a specific bovine adapted serotype. Asymptomatic infections are very common and outbreaks generally occur associated with concomitant undefined stress factors which probably initiate the disease. Adult healthy carriers are a reservoir of the disease (Martel and Moulin, 1983; Sherwood, 1982).

Coccidiosis in calves is a sporadic, severe disease. The animals are generally affected between 1 month to 1 year of life. Two species are commonly involved: Eimeria bovis agent of non-haemorrhagic diarrhoea and, E. zuernii which provokes haemorrhagic scours; E. auburnensis and E. subspherica are also associated with outbreaks of the disease but occur less frequently. E. zuernii causes a very serious haemorrhagic enteritis of the colon and caecum (Yvoré, Naciri and Esnault, 1983).

Other bacteria such as Clostridium perfringens type A, Clostridium sordellii, Actinobacillus lignieresii, Fusobacterium necrophorum and Aeromonas hydrophila (Al-Mashat and Taylor, 1983a) have been isolated from enteric lesions of cattle but they have not been proved to be a frequent cause of enteric disease in calves.

The campylobacters, once included in the genus Vibrio but now separated from it, are a heterologous group of bacteria composed of different species, subspecies and many serogroups - with many of the described microorganisms still unclassified or with an uncertain taxonomic position. The reason for this is that it is only during recent years that improved reliable bacteriological methods have been developed which have allowed the isolation of such bacteria from faeces and their characterization and classification in the laboratory.

More than fifty years ago, when the majority of the now common enteropathogens of cattle were still unknown, Jones and Little (1931a and b) isolated from cases of enteritis in calves over 2 weeks old and from cows suffering from winter dysentery, a bacterium which they described as Vibrio jejuni - possibly Campylobacter jejuni - and after performing some experimental work with conventional animals they concluded that the vibrios were the aetiological agents of the disease. More recently, Al-Mashat and Taylor (1980a and b, 1981, 1983b) isolated campylobacters of different species from enteric lesions of cattle and orally inoculated conventional bovines in a series of experiments and found that the disease produced was very much like the original description of Jones and Little (1931a and b). Nevertheless, because these experiments were carried out in conventional animals they had many of the limitations of the previous

works: the interference of the complex microbial population of the gut and the inevitable presence of many other potential pathogens that might have a synergic effect on the development of the disease. Moreover, other research work carried out in other animal species, even with gnotobiotic animals, has been unsuccessful in producing enteric disease with similar Campylobacter spp., all of which has contributed doubt about the significance of campylobacters as a cause of enteric disease of young ruminants.

During the last few years campylobacter enteritis has been rediscovered as a "new disease" in human medicine and the sudden importance acquired by this zoonotic disease has initiated rapid progress in various areas of this field of research, this has included new methods of accurate isolation and epidemiological data on a variety of environments and animal species which might be responsible for the transmission of the disease to man. As a consequence of this research, nowadays we find that jejuni/coli group Campylobacters (jejuni/coli GC), formerly described as possible cause of enteric disorders in ruminants, are isolated frequently from the faeces of apparently healthy animals so that the mere recovery of campylobacters of these groups from the faeces of diseased animals should not be necessarily linked with the illness. This recently acquired knowledge has been concentrated on only a particular group of campylobacters so that the occurrence of other species, also frequently encountered in the intestinal tract of different animals, remains largely unknown and even the taxonomic position of some of these species has not yet been determined.

So, due to the present state of the research on campylobacters

as a possible cause of disease in ruminants, the following main objectives were considered for this thesis to contribute to a better understanding of the epidemiology and disease producing potential of this group of bacteria:

- 1) The incidence of different species of Campylobacter in diarrhoeic and non-diarrhoeic neonatal calves and their relationship with other common enteropathogens such as rotavirus, coronavirus, ETEC and Cryptosporidium sp.
- 2) Classification of all the isolated strains according to the current taxonomic criteria and a comparison of them with international type strains and also with strains isolated from other species, especially sheep.
- 3) Selection of some representative strains of the groups more commonly found among bovine faecal isolates from cases of neonatal diarrhoea and use of these to inoculate gnotobiotic calves and gnotobiotic lambs as a model for the disease in bovines.

#### Species nomenclature

Before considering the historical review it should be pointed out that the analysis of the literature on different vibriosis (campylobacteriosis) of animals and human beings reveals a great deal of confusion, particularly in the taxonomic field. In order to clarify as far as possible the identification of the Campylobacter spp. dealt with in the text, the species name used by the original author will be followed by the current name in brackets. It will be realised that this second name is the present writer's interpretation

of the data presented and that whereas in some cases there is considerable certainty that the name is correct in other cases there may be some doubt. To indicate those names in which this writer has interpreted the data the species name is followed by the letters HT.

#### Historical background

The first description of a microaerophilic Vibrio (Campylobacter fetus subsp. fetus) was made in a Report of the Departamental Committee appointed by The Board of Agriculture and Fisheries in London (Strachey, Gillespie, McFadyean, Hunting, Nuttall and Stockman, 1909). This report was on the epizootic abortion of cattle due to the "Bacillus of cattle abortion" (Brucella abortus) based on the work of McFadyean and Stockman (1909) and described briefly: "a totally different microbe - a vibrio - has been repeatedly isolated from outbreaks of abortion in ewes, and has been successfully employed at the laboratory to experimentally infect other ewes..." Later, subsequent reports (McFadyean, Devonshire, Strachie, Stockman, Anstruther, Alison, Lane Fox, Hunting and Nuttall, 1913; McFadyean and Stockman, 1913) described the vibrio and the production of abortion in ewes by oral, intravenous or intravaginal inoculation of fresh cultures or infecting material containing living vibrios. Although McFadyean and Stockman (1913) gave a complete description of the bacterium they did not name it because at that time they were awaiting a final classification of spirillar microorganisms.

Smith (1918 and 1919) isolated a microaerophilic "spirilla" from

bovine aborted fetuses, suspecting that this microorganism was closely related to the one reported by McFadyean and Stockman (1909) and reported the successful reproduction of the disease by intravenous (i/v) inoculation of pregnant cows with the vibrios. Smith and Taylor (1919) studied 24 of these vibrio strains and arrived at the conclusion that all of them except one were bacteriologically and serologically similar and named them Vibrio fetus (Campylobacter fetus subsp. venerealis HT).

Later on, vibrios were associated with a different disease of cattle. Smith and Orcutt (1927) called attention to the occasional demonstration of vibrios in the intestinal tract of young calves and speculated about the possibility that these vibrios "might represent a different group possibly associated with intestinal inflammation." One of the strains that they studied had been isolated from the spleen of a scouring calf; interestingly, the mucosa of the large intestine of this animal was overlaid with stringy elastic masses of mucus. Comparative serological studies between calf and fetal V. fetus isolates demonstrated a close relationship among them (but not identity) with only one faecal isolate serologically different. Jones and Little (1931a) described an epidemic form of diarrhoea of adults cows known as "winter scours" because of its seasonal occurrence and characterized by little fever, dark brown or blackish brown liquid faeces ("black scours") often containing mucus and blood and, a marked suppression of the milk secretion. The same investigators (Jones and Little, 1931b) also cited another disease of calves over 2 weeks old characterized by soft or gummy faeces frequently containing blood and mucus. They concluded that in both diseases the aetiological

agent was a microaerophilic vibrio which they designated Vibrio jejuni (Campylobacter jejuni)<sup>HT</sup> (Jones, Orcutt and Little, 1931<sup>a</sup>) supporting this fact by field experiments with cows and calves in which they were able to reproduce the disease either by oral inoculation of a small quantity of intestinal contents or by V. jejuni (C. jejuni) cultures (Jones and Little, 1931a and b; Jones, Little and Orcutt, 1932). The disease they usually found was mild with slight clinical manifestations and although they observed relapses the disease they described was, in general, self-limiting and the animal recovered after a short course which varied between hours to 4 or 5 days. They always found the lesions localized in the small intestine and abomasum and described them as catarrhal inflammation. They believed that the V. jejuni (C. jejuni) that caused disease in calves, although culturally indistinguishable from the one which caused "winter scours", was far more pathogenic for younger animals (Jones and Little, 1931a and b; Jones, Little and Orcutt, 1932; Jones, 1933).

Years later, in 1944, Doyle incriminated microaerophilic vibrios as a cause of swine dysentery, a disease characterized by "pathological changes limited largely, if not entirely, to the colon". He also noted that the colon was the only organ found to be capable of producing the disease when fed to susceptible animals and observed that when the silver nitrate impregnation method was used to examine sections of the affected colon, numerous vibrios were consistently detected deep in the lumen of crypts and, at times, invading the tissues. From mucosal scrapings of the colon of hogs with dysentery he isolated a microaerophilic vibrio which later on he described and



named Vibrio coli (Doyle, 1948). This organism is now considered to be Campylobacter coli although the description of Doyle's strains differs from present isolates in being unable to reduce nitrate. He deduced that V. coli could be the aetiological agent of this disease after 5 successful experiments in which he reproduced the condition in 50 pigs orally infected with pure cultures of vibrios mixed with gastric mucin (Doyle, 1948).

Jones, Little and Orcutt (1932) were the first researchers who drew attention to the possible importance of vibrios in human disease when they stressed: "It is not impossible that vibrios similar in certain respects to those which we have described may play some part in clinically similar diseases of unknown aetiology in man, especially as the usual methods employed in the investigation of such diseases would not lead to their detection". An outbreak of acute gastroenteritis which happened 14 years later proved that this statement was true (Levy, 1946). The infection affected 357 out of 6019 inmates of two penal institutions; the symptoms were nausea, vomiting, abdominal cramps, fever, headache, general malaise and prostration and the illness usually lasted for 3-5 days. Macroscopically, the excreta consisted of watery stools with some mucoid substance which contained when examined microscopically in many cases almost pure cultures of vibrio. These observations led to a search for vibrios and the isolation from blood and faeces of a vibrio very similar to the one described as V. jejuni by Jones, Orcutt and Little (1931) (C. jejuni). Raw milk was suspected of being the source of the outbreak but attempts to substantiate this by culture were not carried out because of the lack of suitable media for recovery from milk. The following year,



Vinzent, Dumas and Picard (1947) isolated a vibrio from a pregnant woman who after suffering cough, fever and headache for 5 weeks delivered a premature stillborn infant and a placenta infected with a vibrio which resembled Vibrio fetus (Campylobacter fetus subsp. fetus HT). From then on, other sporadic human cases were reported (Ward, 1948; Vinzent, Delarue and Herbert, 1950; Auguste, Buttiaux and Tacquet, 1954; Thibault, Gaillard, Second and Chatelain, 1955).

Florent (1953) discovered a new species, Vibrio bubulus (Campylobacter sputorum subsp. bubulus), isolated from the sperm of bulls and the vagina of cows, and he considered it as a saprophytic vibrio that should be differentiated from the pathogenic V. fetus. Bryner and Frank (1955) corroborated Florent's findings and described two types of Vibrio in the bovine genital tract: the catalase positive related with abortion and the non-pathogenic catalase negative vibrios. Akkermans, Terpstra and van Waveren (1956) divided the vibrios from bovine origin in three groups and later Mitscherlich and Liess (1958), by means of complement-fixation tests, divided the vibrios isolated from cattle and sheep into four groups:

- I) V. fetus (C. fetus subsp. venerealis HT) which causes genital vibriosis in cows
- II) V. fetus (C. fetus subsp. fetus HT) which causes sporadic abortion in cows, enteritis in calves and genital vibriosis in ewes
- III) V. bubulus (C. sputorum subsp. bubulus HT) isolated from vaginal secretion in cows, and
- IV) Vibrio spp. (Campylobacter spp.) isolated from preputial washing of bulls.

Florent (1959a) stated that bovines have two types of vibriosis, each caused by a distinct aetiological agent which lives in two different habitats. One disease, the "enzootic sterility" characterized by temporary infertility and occasional abortions, is venereally transmitted and is produced by Vibrio fetus venerealis (Campylobacter fetus subsp. venerealis HT). The other, the "sporadic abortion" not related with infertility, is orally transmitted and is caused by Vibrio fetus intestinalis (Campylobacter fetus subsp. fetus HT). Florent (1959b) classified four groups of vibrios which do not ferment carbohydrates:

- I) Vibrio fetus venerealis (C. fetus subsp. venerealis HT) and,  
V. fetus intestinalis (C. fetus subsp. fetus HT)
- II) V. bubulus (C. sputorum subsp. bubulus HT)
- III) Aerobic vibrios group (possibly some of the aerotolerant campylobacter-like organism of Neill, Ellis and O'Brien, 1979 HT)
- IV) Coli-jejuni group (jejuni/coli GC HT)

It is worth noting that this classification was the first to cite the major groups that we recognize nowadays. Later, Bryner, Frank and O'Berry (1962) and Mohanty, Plumer and Faber (1962) proposed two classification schemes differentiating 3 types of bovine Vibrio fetus.

Hofstad (1956) isolated a vibrio from chickens with infectious hepatitis and Peckhan (1958) reproduced the disease by inoculation of chickens with the bacterium and suggested that the disease should be called vibronic hepatitis. These vibrios (jejuni/coli GC HT) are present in faeces and intestinal contents of infected poultry (Peckhan, 1972). Truscott, Connell, Ferguson and Wills (1960)

isolated a microaerophilic bacterium (jejuni/coli GC HT) from turkey poultts affected with bluecomb (transmissible enteritis). Truscott and Morin (1964) classified it as a vibrio and were able to reproduce the disease by oral infection of susceptible poultts.

Kuzdas and Morse (1956) studying several microaerophilic and aerobic vibrios of different origin, found that a strain of Vibrio jejuni (jejuni/coli GC HT) isolated from an outbreak of bovine dysentery, was able to grow at 45°C. King (1957) compared human and animal microaerophilic vibrios and concluded that three species should be considered: Vibrio fetus (C. fetus subsp. fetus HT), "related vibrios" (jejuni/coli GC HT) and, V. bubulus (C. sputorum subsp. bubulus HT). She discovered that V. fetus and V. bubulus grew at 25°C or 37°C with very little or no growth at 42°C while the "related vibrios" failed to grow at 25°C and grew more luxuriantly at 42°C than at 37°C. King initially studied 4 strains of "related vibrios" isolated from children, three of them with diarrhoea, and stated that this microorganism might be closely related to V. jejuni (jejuni/coli GC HT) and important in childhood diarrhoea of unknown aetiology. Peckhan (1958) sent the bacterium of the vibronic hepatitis of chickens to King who found that it shared many characteristics of the human "related vibrios". Later, King (1962) reported the isolation of 7 other strains of "related vibrios"; one was isolated from a chicken farmer who could have acquired the infection from the birds. She compared a strain of those described as Vibrio coli isolated from cases of swine dysentery, finding it very similar to the human "related vibrios". As a final conclusion, she suggested that the causative agent of vibronic hepatitis in

chickens, winter dysentery in cattle, vibrionic enteritis in calves, swine dysentery and vibrionic dysentery in humans could be the same. The same bacteria have been called until then V. jejuni in cattle and V. coli in swine and, for that reason, she considered it unwise to give a new species name to the human or avian strains.

Russell (1955) described two cases of profuse watery scouring in hoggets observing numerous vibrios in carbol-fuchsin smears of intestinal mucosa and faecal material from the jejunum and ileum in one case, and of the large intestine in the other. Electron micrographs showed a vibrio half the length of V. fetus. Intra-ruminal inoculation of two hoggets with macerated intestines and contents failed to produce scouring.

Bryans, Smith and Baker (1960) described the isolation of two new "Vibrio spp." strains (jejuni/coli GC HT) from ovine aborted fetuses. Bryans and Smith (1960) compared several bovine and ovine strains with strains isolated from cases of avian infectious hepatitis and found that 2 bovine and 7 ovine strains were indistinguishable from the avian vibrios. Bryans and Shephard (1961) performed oral experimental infections in lambs with cultures of one ovine "Vibrio spp." strain and detected watery diarrhoea in 3 out of 11 infected animals; diarrhoea occurred 24 to 72 hours post-inoculation (PI) and lasted for 3 to 9 days. Later on, several studies were made on strains from various sources to determine whether the vibrios were part of the intestinal flora, the distribution of the different species and the ability of strains to colonize. Bryner, O'Berry and Frank (1964) demonstrated that V. fetus type 1 and subtype 1 (C. fetus subsp. venerealis HT) were not able to colonize the

intestinal tract of orally infected bovines while type 2 (C. fetus subsp. fetus HT) was recovered from duodenum, faeces and livers of inoculated animals. Firehammer, Lovelace and Hawkins (1962) found that different strains of V. fetus (C. fetus subsp. fetus HT) were frequently encountered in ovine livers and gallbladders not only in pregnant ewes which aborted, but also in asymptomatic carriers. Intra-ruminal or i/v inoculation of pregnant ewes with V. fetus (C. fetus subsp. fetus HT) isolated from bile, generally produced abortion (Firehammer and Hawkins, 1964). Smibert (1965a) referred to the frequent isolation of strains of microaerophilic vibrios similar to Vibrio fetus var. intestinalis (jejuni/coli GC HT) from faeces and intestinal contents of clinically normal sheep and deduced that they were indigenous to the intestinal flora. Smibert (1965b) observed that these vibrios were very similar to the human "related vibrios" of King (1957 and 1962), the avian vibrios of Truscott and Morin (1964), the ovine "Vibrio spp." of Bryans, Smith and Baker (1960) and the vibrios of sporadic abortion of Florent (1960). Firehammer (1965) demonstrated the colonization of the ovine intestinal tract by Vibrio fetus intestinalis (C. fetus subsp. fetus HT) and discovered a new species, Vibrio fecalis ("Campylobacter fecalis"), which he described as resembling a "catalase positive Vibrio bubulus".

In 1963, Sebald and Véron suggested that the microaerophilic Vibrio fetus (Smith and Taylor, 1919) and Vibrio sputorum (Prévot, 1940) be removed from the genus Vibrio and proposed a new genus, the Campylobacter. The only shared property between the genus Vibrio and Campylobacter - curved cells - is now recognized to be a

a characteristic of dubious taxonomic value. Later on, Véron and Chatelain (1973) re-arranged and re-classified the majority of the species of the new genus and this taxonomic study has now been accepted as valid in the "Approved List of Bacterial Names" (Skerman, McGowan and Sneath, 1980). The Véron and Chatelain nomenclature will be used in this thesis, except when the Campylobacter sp. described has not been included under this taxonomic scheme. Nevertheless, another campylobacter nomenclature has been extensively used during recent years (Smibert, 1974). In order to clarify the subject as far as possibly both nomenclatures will be compared in a brief taxonomic review.

The analysis of the principal historic events shows the great confusion which existed in the taxonomy of the present Campylobacter genus. Different investigators cited the same microorganisms under different denominations while others have cited more than one species under the same denomination. Campylobacters are complex to study because there is a diversity of species and serotypes, pathogenic or not, sharing the same habitat and several of them can be isolated from species as dissimilar as a mammal or a bird. The ignorance of these facts ensured that some investigators named "new" species only on the basis of the origin of the isolation.

This confusion has been compounded by a number of other features of these organisms, importantly the lack of suitable simple laboratory tests which clearly separate the species. Initial studies concentrated on the campylobacters associated with abortion and as at that time these bacteria could only be recovered - due to the lack of suitable inhibitory media - from relatively "sterile" sites, knowledge of the

epidemiology and distribution was fragmentary.

An example of the influence exerted by the isolation methods in the study of campylobacters is the comparison between two works of isolation of these bacteria from ovine faeces. Firehammer (1965) using a selective culture medium, could isolate C. fetus subsp. fetus and discovered "C. fecalis" while Smibert (1965b) employing filtration isolated jejuni/coli GC.

The early historic development of research on microaerophilic vibrios cannot be understood without analyzing events in different areas of veterinary and medical sciences as a whole. Due to the great amount of work published during the last few years I consider that, after the creation of the Campylobacter genus it is convenient to describe the current state of campylobacter taxonomy. Then the review will continue with a brief sketch of the development of knowledge of these bacteria in human medicine as this area has developed faster than any other. Finally, the literature relating to the campylobacters as a cause of enteric disease in animals, in particular ruminants, will be assessed.

### Taxonomy and Biotyping

The genus Campylobacter (from the Greek words "campylo" meaning curved and "bacter" meaning rod) was created by Sebald and Véron (1963) to include the microaerophilic non-fermentative organisms which have DNA with a G + C content between 29 to 36 mol per cent. The genus Vibrio, on the other hand, comprises bacteria which ferment glucose and contain DNA with G + C content between 40 and 53 mol



per cent (Véron and Chatelain, 1973). The genus Campylobacter and the genus Spirillum together form the family Spirillaceae because of their morphological and physiological resemblance (Véron, 1966).

Up to now, this genus has contained four officially recognized species: Campylobacter fetus, Campylobacter jejuni, Campylobacter coli and Campylobacter sputorum (Skerman, McGowan and Sneath, 1980). Besides, C. fetus and C. sputorum are each subdivided into two subspecies, C. fetus subsp. fetus, C. fetus subsp. venerealis, C. sputorum subsp. sputorum and C. sputorum subsp. bubulus (Table 1.1). One other taxonomic scheme has been extensively used, especially in the United States and, although it is still quoted in some works, in general, bacteriologists are shifting to the official terminology. This former taxonomic grouping was published in Bergey's Manual of Determinative Bacteriology 8th ed. (Smibert, 1974) and in it the genus is composed of three species: C. fetus, C. sputorum and "C. fecalis". C. fetus and C. sputorum are each subdivided into three and two subspecies respectively. C. fetus includes the subspecies fetus, intestinalis and jejuni and C. sputorum the subspecies sputorum and bubulus (Table 1.2). Comparing both taxonomic schemes some confusion exists among the denomination of four of the officially recognized species (Table 1.3). For instance, C. fetus subsp. fetus of Véron and Chatelain is equivalent to C. fetus subsp. intestinalis of Smibert, C. fetus subsp. venerealis of Véron and Chatelain is equivalent to C. fetus subsp. fetus of Smibert and the separate species C. jejuni and C. coli of Véron and Chatelain have been combined into one subspecies of C. fetus by Smibert (Table 1.3). For that reason, in most of the published works when C. fetus subsp.



jejuni is described it is not possible to know if the bacterium is C. jejuni or C. coli so, in this thesis this microorganism will be generically referred to as jejuni/coli GC.

Unfortunately, neither scheme is complete because during the last few years new microorganisms, previously undescribed, have been discovered. The officially approved list of microorganisms only contains those described by Véron and Chatelain in 1973 at a time when very few strains were available for taxonomic studies. So, for the practical purposes of this thesis the official terminology will be used but, the new species will also be included with their definitive or provisional names. When a valid description of the species or subspecies had been published the name will be mentioned as a recognized species although not included in the official list. In other cases, when the species or subspecies cited do not have a valid description up to the end of 1983 and their position is still uncertain they will be expressed with their provisional names between quotation marks. A provisional current taxonomical grouping of species is given in Table 1.4.

One of the major problems of the taxonomy of this genus is the lack of suitable laboratory phenotypic tests to correlate with genotypical characters of the species. Many of the tests considered to be accurate a few years ago are now regarded as unreliable. For instance, the ability to grow in media containing 1 per cent of glycine was estimated to be crucial in the separation of subspecies C. fetus subsp. fetus (grower) from C. fetus subsp. venerealis (non-grower). Nevertheless, glycine tolerance of a single strain of C. fetus can differ in the same basal medium or in different basal

media (Allsup and Hunter, 1973; Harvey and Greenwood, 1983). Ten successive passages through a medium containing 0.5 per cent of glycine produced an adaptation of the bacterium to the aminoacid in 2 out of 4 Vibrio fetus type 1 venerealis (C. fetus subsp. venerealis) and in all 4 subtype 1 strains tested which subsequently were able to grow in 1 per cent glycine (Florent, 1963). Furthermore, Chang and Ogg (1971) demonstrated phage-mediated transduction of glycine tolerance from donor to recipient strains. C. fetus subsp. venerealis was considered as a separate subspecies mainly because it has a high degree of parasitism and a restricted ecological niche in the bovine genital tract being unable to multiply in the intestinal environment. This microorganism could be considered as a very closely related defective mutant of C. fetus subsp. fetus which is regarded as being the wild strain (Véron and Chatelain, 1973). Intermediate strains have been described by Florent (1963) and El Azhary (1968) as "intermediate group" or "Vibrio fetus subsp. intermedius" respectively; these strains are adapted in cattle to either the genital or intestinal tract. In addition, strains of C. fetus subsp. fetus are able to multiply and persist for long periods in the genital tract of cattle (Park, Munro, Melrose and Stewart, 1962; Agumbah and Ogaa, 1979). On the other hand, besides the superficial similarity of the organism they also have closely related DNA values which do not justify the division into the present subspecies (Harvey and Greenwood, 1983). In fact a type strain of C. fetus subsp. venerealis was 100 per cent homologous with the type strain of C. fetus (Belland and Trust, 1982). Comparing standard strains Karmali, Allen and

Fleming (1981) proposed that C. fetus subsp. venerealis can be distinguished from C. fetus subsp. fetus by its larger size, higher mean values of average wave length and amplitude of spiral forms. Nevertheless, this is neither a practical test nor easy to perform and it is not known if it could be used to differentiate the sub-species in a wide range of strains. Recently, by means of a new staining method to demonstrate flagella which is almost as simple and quick to perform as a Gram stain (Kodaka, Armfield, Lombard and Dowell, 1982) it was found that cultures of C. fetus subsp. venerealis and the variety "intermedius" were unique in containing up to 10 per cent lophotrichate cells, with up to five flagella at one pole, whereas C. fetus subsp. fetus is monotrichate and only occasional cells have two flagella at one pole (Skirrow, Purdham and Benjamin, 1983).

Recently, "C. hyointestinalis" (CHI) isolated from pigs (Gebhart, Ward, Chang and Kurtz, 1983) was proposed as a new species, similar to C. fetus in all respects but able to produce hydrogen sulphide ( $H_2S$ ) in insensitive media such as Triple Sugar Iron agar (TSI). The taxonomic relationship between CHI and organisms previously identified as C. fetus is not clear. Up to now, no studies have been done to show if hydrogen sulphide producing C. fetus strains are genetically different from the non-producers.

C. laridis (formerly Nalidixic Acid-Resistant Thermophilic Campylobacters: NARTC) are phenotypically and genotypically different from other campylobacters (Benjamin, Leaper, Owen and Skirrow, 1983; Harvey and Greenwood, 1983). The phenotypical characters of C. laridis are closest to those of C. coli although some are shared

with C. jejuni and C. fetus subsp. fetus but, genotypically, they are less than 20 per cent related to any other campylobacter.

The jejuni/coli GC have been called thermophilic campylobacters for their ability to grow luxuriantly at 42°C or 43°C. Despite this name this characteristic is shared by many other strains, C. laridis, "C. fecalis", some CHI and even some C. fetus subsp. fetus. So the term thermophilic campylobacters will not be used in this thesis to name this group of microorganisms. At present, C. jejuni and C. coli can be phenotypically distinguished by means of a rapid hippurate hydrolysis test which correlates well but not completely with genotypical classification (Harvey and Greenwood, 1983). The growth in media containing 1/33,000 Brilliant Green has been formerly employed to differentiate between C. jejuni strains (non-growers) and C. coli (growers) (El Azhary, 1968; Véron and Chatelain, 1973) but at present this test is considered useless to identify these species (Hasselbach, 1982). The C. jejuni can be divided into two biotypes by means of H<sub>2</sub>S production in iron-metabisulfite-pyruvate medium (Skirrow and Benjamin, 1980a). Both biotypes are genetically similar with an average relationship of 85 per cent (Leaper and Owen, 1982). There is no doubt to-day that, genetically, C. jejuni and C. coli are two separated species (Owen and Leaper, 1981; Leaper and Owen, 1982; Belland and Trust, 1982; Harvey and Greenwood, 1983) although some of them share common antigenic components detectable by the serotyping technique of Penner and Hennessy (1980). Other tests have been proposed to biotype jejuni/coli GC strains but their usefulness has not been proved (Holländer, 1982; Hébert, Hollis, Weaver, Lambert, Blaser and Moss, 1982).

C. sputorum species are catalase negative campylobacters. C. sputorum subsp. sputorum (Prévot, 1940) and C. concisus (Tanner, Badger, Lai, Listgarten, Visconti and Socransky, 1981) are two genetically unrelated species isolated from the bucal cavity of humans. C. sputorum subsp. mucosalis has been isolated from porcine proliferative enteropathies and oral cavity of pigs and is similar to the oral human Vibrio spp. in its hydrogen dependance yet the G + C content of the DNA supports its inclusion in the genus Campylobacter. This subspecies contains an unusual C type cytochrome and is serologically distinct from other campylobacters (Lawson, Leaver, Pettigrew and Rowland, 1981).

C. sputorum subsp. bubulus has been isolated from the genital and intestinal tract of ruminants by Florent (1953 and 1959b). Firehammer (1965) isolated "C. fecalis" from the intestinal tract of sheep and described it as resembling a catalase positive C. sputorum subsp. bubulus. Later on, Smibert (1974) classified "C. fecalis" and C. sputorum as two separate different species. Nevertheless, the official list of approved bacterial names did not include "C. fecalis" (Skerman, McGowan and Sneath, 1980). Skirrow, Purdham and Benjamin (1983) discovered that C. sputorum subsp. bubulus and "C. fecalis" have a similar and distinct morphology characterized by unusually long flagella (monotrichate) with a short wave length relative to cell wave length. Recently, Harvey and Greenwood (1983) found that reference strains of "C. fecalis" are 75 per cent genetically related to one strain of C. sputorum subsp. bubulus.

Campylobacters that are able to grow aerobically have been isolated from bovine fetuses (Florent, 1959b; Walsh and White, 1968)

although superficially described. More recently, similar bacteria have been isolated from bovine and porcine fetuses (Neill, Ellis and O'Brien, 1978 and 1979), bovine mastitis (Logan, Neill and Mackie, 1982; Neill, Mackie and Logan, 1982) and bovine preputial sheath washings. Up to now, this group of microorganisms has not been isolated from the intestinal tract. These bacteria belong to the Campylobacter genus according to the DNA base G + C which lies within the span of 29 to 34 mol per cent. Nevertheless, they differ from any other species of the genus by their ability to grow aerobically - aerotolerant campylobacters - and also at 15°C - psychrophilic campylobacters - (Neill, Ellis and O'Brien, 1979; Neill, O'Brien and Ellis, 1983). Another unique characteristic is the production of cytoplasmatic inclusions (Harvey and Greenwood, 1983).

The campylobacters isolated from laboratory mice are more fastidious in their growth requirements than any other campylobacters tested, are difficult to subculture and do not grow in broth medium (Fox, Zanotti and Jordan, 1981; Harvey and Greenwood, 1983). Some of these strains have been studied in more detail and it has been shown that they form a separate DNA relationship group. Other non-grower campylobacters have been isolated from hamsters (Lawson, Personal Communication, 1983).

A further new species "C. pyloridis" associated with cases of human gastritis and duodenal ulcers conforms<sup>to</sup> the G + C contents of the Campylobacter genus but the presence of unipolar sheathed flagella differentiates it from any other member of the genus (Marshall and Warren, 1983; Warren and Marshall, 1983). A group provisionally called campylobacter-like organisms (CLO's) has been isolated from

human faeces and is phenotypically very similar to jejuni/coli GC although some strains are not able to grow at 42°C. This group of bacteria does not have genetic homology with C. jejuni, C. coli or C. fetus (Fennell, Totten, Quinn, Holmes and Stamm, 1983).

Other two new groups have been described and they also seem to represent new campylobacters. One group with negative or weak catalase activity (CNW), isolated from dog faeces by means of a medium without inhibitors, has a G + C content of 35 mol per cent and is 50 per cent genetically related to C. jejuni and C. coli. They differ from C. jejuni because they grow more slowly at 37°C and less at 42°C, fail to hydrolyse hippurate, are sensitive to cephalothin and very sensitive to 2,3,5 triphenyl-tetrazolium chloride (Sandstedt, Ursing and Walder, 1983). The other group is composed of nalidixic acid resistant faecal strains isolated from pigs and cattle with their G + C content of 35 mol per cent and with less than 20 per cent homology with jejuni/coli GC and is genetically more related to C. fetus.

Besides the DNA studies, the cellular fatty acid and protein profiles have been recently employed for taxonomic purposes. For instance, it has been demonstrated that there is a characteristic distribution of fatty acid among campylobacters and this enables three broad groups to be distinguished: C. fetus subspp., jejuni/coli GC and C. sputorum subspp. In addition, there is evidence which supports the view that C. laridis occupies an intermediate position between C. fetus and C. coli (Curtis, 1983). Krause and Ullman (1983) by means of high performance liquid chromatography detected the production of volatile and non-volatile fatty acids. It was found that C. jejuni



strains generally produce malonic and pyruvic acids while C. coli only pyruvic acid and these two fatty acids are specific for jejuni/coli GC. "C. fecalis" was unique in producing fumaric acid. Examining total protein patterns of strains it was determined that C. sputorum and "C. fecalis" are characterized by a major protein band of an approximate molecular weight of 44,000 daltons, jejuni/coli GC, C. laridis have a major protein band of 39,000 to 41,000 daltons and, C. fetus subsp. have a major protein band of 42,000 daltons. Besides this major band the overall pattern of 40 to 50 bands could also be used to clearly distinguish among many of the species (Costas and Owen, 1983). Lastovica, Kirby, Carr and Robb (1983) obtained different values for major protein bands of C. jejuni (42,000 daltons) and C. coli (44,000 to 45,000 daltons).

The DNA studies, the cellular fatty acid and protein profiles could be useful but not practical for small diagnostic or research laboratories. There is a need for more practical tests to differentiate the species, subspecies and the increasing number of phenotypically similar but genetically distinct new campylobacters. The selection of tests for use in differentiating species should be done choosing only those able to correlate with genotypical differences among this heterogenous group of microorganisms. For instance, coccal transformation and cell size (Karmali, Allen and Fleming, 1981), tolerance to nalidixic acid (Skirrow and Benjamin, 1980b) and, to a lesser extent cephalothin sensitivity (Karmali, De Grandis and Fleming, 1980) are very useful tests which are easy to perform and accurately differentiate campylobacters of the "fetus" and "jejuni/coli" groups. Similarly, the hippurate and the 2,3,5 triphenyl-



tetrazolium chloride (TTC) are easy to do and are useful to distinguish between C. jejuni and C. coli (Skirrow and Benjamin, 1980b). Other tests give variable results within one species and have been used to subdivide species into biotypes. For instance, C. jejuni has been divided into four biotypes and C. coli and C. laridis into two biotypes respectively (Lior, 1983) by means of the combination of two modified tests: hydrogen sulphide production in iron-metabisulfite-pyruvate medium (Skirrow and Benjamin, 1980a) and deoxyribonuclease production (Hébert, Hollis, Weaver, Lambert, Blaser and Moss, 1982). Up to now, the epidemiological value of this biotyping scheme has to be demonstrated.

It is evident from this taxonomic review that the nomenclature of species and subspecies of this genus is still confused. The uncertainty in the taxonomic position of some members of this genus is in part due to the overwhelming discovery of new species but mainly it is closely linked to the lack of suitable tests to identify the species and subspecies. Besides, our inability to detect in vitro attributes of pathogenicity among indistinguishable similar strains makes things more difficult. The research done in the last few years has outlined a very heterogenous group of bacteria, it is likely that many new species will be added to the list and radical changes in the present official nomenclature should be expected.

#### Serotyping schemes

The first serotyping schemes have involved strains isolated from bovine and ovine abortions so, in general, they have mainly

considered the study of strains of C. fetus subspecies and, to a lesser extent some jejuni/coli GC and C. sputorum subsp. bubulus. Numerous authors have proposed their own classification based on different methods ignoring sometimes the work of their predecessors so, in many cases it is not possible to establish any connection between these serological schemes. Old classifications which have no practical use nowadays will not be discussed here. A recent review compares some of the serological schemes used in the past (García, Eaglesome and Rigby, 1983).

Berg, Jutila and Firehammer's (1971) serological classification and biotyping scheme was described as follows:

- 1) Group A-1 (serotype A-biotype 1). Glycine and H<sub>2</sub>S negative.
- 2) Group A-sub. 1 (serotype A-biosubtype 1). Glycine negative, H<sub>2</sub>S positive.
- 3) Group A-2 (serotype A-biotype 2). Glycine and H<sub>2</sub>S positive.
- 4) Group B (serotype B).
- 5) Group C (serotype C).

By means of agglutination tests on campylobacters isolated from bovine abortions, they demonstrated three thermostable O antigens (A, B and C) and seven thermolabile antigens. The serotype A is composed by C. fetus subsp. venerealis and C. fetus subsp. fetus; the serotype B by C. fetus subsp. fetus and the serotype C corresponds to the description of jejuni/coli GC. The seven heat labile antigens were reported in C. fetus in which one to five can be found in one strain. The group A-1, A-sub.1 and B share common thermolabile antigens. The C thermostable antigen of the jejuni/coli GC was common to all the strains which could be tested, and some strains auto-

agglutinated after heating. "C. fecalis" and C. sputorum subsp. bubulus were considered biochemically unrelated and not included in the scheme. Berg's groups A-1 and A-sub.1 strains were considered the only two groups not able to survive in the intestinal tract of bovines (Bryner, O'Berry and Frank, 1964) and were proposed as causative agents of bovine infertility (C. fetus subsp. venerealis). So, the subspecies venerealis and fetus are not only genetically related but up to now, phenotypically indistinguishable and also some strains are closely antigenically related. This serological scheme was later corroborated by means of co-agglutination and immunoelectrophoresis techniques (Kosunen, Danielsson and Kjellander, 1980).

Serological studies by means of complement fixation tests have determined that C. sputorum subsp. bubulus is serologically distinct from C. fetus or jejuni/coli GC (Mitscherlich and Liess, 1958). Up to now, no serological studies have been done on C. sputorum subsp. bubulus, "C. fecalis" or "C. hyointestinalis".

C. sputorum subsp. mucosalis is serologically different from other members of the species C. sputorum or catalase positive campylobacters. By means of agglutination tests, two and possibly three distinct serogroups of organisms appear to be involved in the porcine adenomatosis complex: serovar A, B and possibly C (Lawson, Leaver, Pettigrew and Rowland, 1981). Within serotype A strains, a variety of different common surface antigens were detected and no distinctive pattern could be demonstrated (Lawson, Rowland and Roberts, 1977).

Agglutination tests have been useful for the serotyping of C.

fetus and C. sputorum species but with jejuni/coli GC other techniques were developed or the agglutination techniques modified because auto-agglutination of a high percentage of strains made these schemes difficult to interpret. The Berg, Jutila and Firehammer's (1971) scheme can be used for identification of campylobacter subspecies associated with abortions but it would not be useful as an epidemiological tool to determine the origin of the strains, although the common O antigen C appears in abortion strains of jejuni/coli GC, its distribution in non-abortigenic human strains is very much less clear (Abbott, Dale, Eldridge, Jones and Sutcliffe, 1980). Other works also corroborate that heat stable O antigens are often shared and using certain methods of preparation they commonly auto-agglutinate even when the original suspension is smooth (Butzler and Skirrow, 1979). Butzler (1978) reported that auto-agglutinability occurred in 50 per cent of the cases with living organisms and that treatment with 0.5 per cent formalin reduced but did not eliminate auto-agglutination. Bryner, Ritchie and Foley (1982) also found that auto-agglutination interferes with sero-testing most strains of C. jejuni. Examination of cultures incubated for 48 hours at 43°C reveals by EM more than 40 per cent of disrupted cells which have a mantle of DNA-like material and also protein-like material covering the flagella. Treatment with DNA-ase digestion reduced auto-agglutination but not completely. Young cultures incubated 18 hours at 43°C have few disrupted cells and minimal auto-agglutination. The O-antisera normally agglutinate, often to high titre, with heterologous as well as homologous suspensions and, in order to partially eliminate cross reactions sera should be

absorbed with heterologous strains (Abbott, Dale, Eldridge, Jones and Sutcliffe, 1980). For that reason, different serological schemes have been developed in different parts of the world using OH rabbit hyperimmune sera and slide agglutination tests. In Canada, a serotyping scheme for C. jejuni and C. coli has been extended to 53 serogroups and it is based on slide agglutination of live bacteria with whole cell antisera absorbed with homologous heated and heterologous unheated cross-reactive antigens. In this scheme, 2,002 out of 2,158 (92.8 per cent) cultures of jejuni/coli GC of different origins were found typable and 156 (7.2 per cent) untypable due to roughness. One major improvement in this technique which reduces the number of auto-agglutinating, rubbery sticky strains of C. jejuni is the treatment with DNA-ase which does not affect the serological specificities and does allow the serotyping of otherwise untypable strains (Lior, Woodward, Edgar, Laroche and Gill, 1982; Lior, Woodward, Laroche, Lacroix and Edgar, 1983). In Israel, using a very similar scheme of 58 serogroups of OH antisera and slide agglutination of fresh cultures, it was found that 525 (93.8 per cent) out of 560 were typable, 18 (3.2 per cent) did not react with any sera and 17 (3 per cent) were auto-agglutinable (Rogol and Sechter, 1983). In Japan, slide agglutination tests using formalin-treated heat-labile antigens instead of fresh cultures were used initially and they were later replaced by cell antigen heated at 100°C for 60 minutes. By means of this scheme, some 887 (79.8 per cent) out of 1,111 human C. jejuni isolates and 78 (55.7 per cent) out of 140 isolates of animal origin were typed. It is interesting to note that in comparative studies half of reference strains used

in other serotyping schemes remained untypable by the Japanese system suggesting a regional distribution of different serotypes (Itoh, Saito, Yanagawa, Takahashi, Kai, Inaba, Takano and Ohashi, 1983). In general, under these schemes some C. jejuni and C. coli belong to similar serogroups, showing that they may share heat-labile superficial antigens.

In some veterinary (Newsam and George, 1967) and human (Bokkenheuser, 1972) serological studies on C. fetus strains, the passive haemagglutination test (HA) proved to be more sensitive than the bacterial agglutination test. Later on, Penner and Hennessy (1980) and Lauwers, Vlaes and Butzler (1981) adopted this technique and developed serotyping systems which use passive haemagglutination techniques with heat-stable soluble antigens and crude, unabsorbed sera produced against whole cells. The antigens are extracted by heating bacterial suspensions at 100°C for 1 hour and then used to sensitize sheep erythrocytes. These antigens could also be extracted by exposure to ethylenediaminetetraacetic acid (EDTA), techniques which are known to produce lipopolysaccharide (O) antigens from Gram negative species (Penner and Hennessy, 1980). Nowadays, this last scheme is the most widely used and numerous laboratories have adopted it, mainly because it completely eliminated the problem of auto-agglutination and it is easy to implement and read. This scheme has been expanded and, up to the present, it contains 42 antisera for C. jejuni and 18 for C. coli (Penner, Hennessy, Congi and Pearson, 1983). Cross reactions between these species are uncommon and only four (0.2 per cent) of 2,025 hippurate positive C. jejuni isolates reacted to C. coli antisera and 12 (4.3 per cent) of 282 C. coli

reacted to C. jejuni antisera (Penner, Hennessy and Congi, 1983).

Recently, The Center for Disease Control in USA compared the Penner and Hennessy HA technique with Lior's slide agglutination method, serotyping in parallel human and animal isolates of jejuni/coli GC. Most human strains were typable by both schemes but the Penner and Hennessy system typed a greater percentage of the non-human isolates. As expected, there was no complete correlation between the serotypes in the two schemes since they detect different antigens. Comparison of the two systems is yet incomplete but it seems that HA antigens may be present with different combinations of heat labile antigens and that the use of the two systems provided more epidemiological data. Certain combinations of Lior and Penner and Hennessy antigens seem to occur (Kaijser and Sjogren, 1983). The Penner and Hennessy system is easier to perform but it has the disadvantage that sometimes the tested strains react to multiple antisera whereas campylobacters tested by Lior's method are more likely to react with only one antiserum (Patton, Barret and Morris, 1983).

Penner and Hennessy's technique has been used to serotype C. jejuni strains from cattle and sheep. Munroe and Prescott (1983) found that 57 per cent of isolates from cattle with diarrhoea and 60 per cent of isolates from healthy cows belonged to 12 serotypes which also occur frequently in human campylobacter enteritis. Other investigators have reported the introduction of the Penner and Hennessy scheme to serotype strains of sheep and cattle but no information about this work has been published up to the end of 1983 (Ansfield and Duffell, 1983; Roberts, 1983). Lior's scheme was also used to serotype C. jejuni and C. coli isolated from slaughtered



cattle and it was found that 81 (78.6 per cent) out of 103 cultures were typed and represented 14 serotypes. Interestingly, 51 out of 100 slaughtered cattle had C. jejuni (50) or C. coli (1) and 31 animals had only one serotype, 16 had two serotypes, 2 had three serotypes and 2 had four serotypes showing that it is very common to find one bovine harbouring more than one serotype. These results provide further evidence that serotypes commonly isolated from human sources are also found in cattle (García, Lior, Stewart, Ruckerbauer and Skljarevski, 1983 and Poster Communication of the Second International Workshop on Campylobacter Infections, Brussels, 1983). Combining Lior's and Penner and Hennessy's typing methods Pearson, Bartlett, Page, Jones, Lander, Lior and Jones (1983) demonstrated that there was a variety of serotypes excreted by a herd of dairy cows and both systems were useful to detect "epidemic" strains responsible for a human milk-borne outbreak.

#### Brief review of recent developments in human campylobacter studies

Jones' (1932) suggestion and King's (1962) hypothesis on the possible implication of microaerophilic vibrio in human disorders of unknown aetiology was only confirmed in the 70s. Until then, researchers reported only occasional isolations from blood or other sterile sites free from competing microorganisms. Until 1969, only 74 cases of human vibriosis were published in the international medical literature, most of them due to Vibrio fetus (Campylobacter fetus subsp. fetus HT) acting as a secondary opportunistic agent of other diseases and only a few cases of "related vibrios" (jejuni/coli GC)



associated with diarrhoea in children (Bokkenheuser, 1970).

El Azhary (1968) was the first to isolate Vibrio fetus intestinalis (C. fetus subsp. fetus HT) and Vibrio coli (jejuni/coli GC) from human faeces. By means of two selective media based on brilliant green and antibiotics and filtration techniques, he was able to isolate Vibrio fetus intestinalis from 2 faeces and Vibrio coli from 3 out of 95 stools taken at random from patients of a clinic. Unfortunately, although these findings were significant, they had no influence on the medical field, perhaps because the subject of his work was mainly related to animal infections.

Later on, Cooper and Slee (1971) were able to isolate Vibrio fetus (jejuni/coli GC) by direct inoculation of faeces onto horse blood agar plates and subsequent application of cephalothindiscs. The following year, Slee (1972) was able to isolate another Vibrio fetus (jejuni/coli GC) using a veterinary method of isolation (Clark, Monsborough and Dufty, 1969) which consisted in culturing supernatants of centrifugates of diluted faeces in a selective medium with antibiotics. They suspected that the microorganisms were part of the intestinal flora but no carriers were detected among 30 patients investigated. Simultaneously, combining filtration techniques with a selective medium, a joint veterinary and medical research team was able to isolate vibrios from the stools of two patients with acute enteritis (Dekeyser, Gossuin-Detrain, Butzler and Sternon, 1972). Butzler, Dekeyser, Gossuin-Detrain and Dehaen (1973) applied the new technique to screen the stools of a large number of patients with and without diarrhoea, to assess the occurrence of the infection. "Related vibrios" were isolated from 41 out of

800 children (5.1 per cent) and 4 out of 100 adults (4 per cent) with diarrhoea. This contrasted with an isolation rate of 1.3 per cent in 1,000 children without diarrhoea. Although these studies clearly demonstrated the widespread occurrence of the jejuni/coli GC in human enteric disorders, the frequency of isolations only changed significantly after Skirrow (1977) developed a new selective culture medium onto which stool specimens could be directly inoculated without any previous filtration. Using this new technique Skirrow (1977) isolated jejuni/coli GC from 57 out of 803 patients with diarrhoea (7.1 per cent) and none from 194 apparently healthy controls.

Skirrow's study generated considerable interest among the medical community and since then many other researchers throughout the world have reported similar findings. Nowadays, in some laboratories, Campylobacter spp. isolations have outnumbered those of Salmonella spp. and Shigella spp. together (Butzler and Skirrow, 1979). For instance, the number of reports of campylobacter isolations to The Communicable Disease Surveillance Centre, Colindale, from England and Wales increased as follows: 6,346 cases in 1978, 9,506 in 1980 and 12,496 in 1981 (Skirrow, 1982). This phenomenon has happened in several countries and the number of campylobacter isolations from the jejuni/coli group increases year after year as more bacteriological laboratories use improved culture media for their isolation. Campylobacter enteritis has been reported in countries of Europe, America, Oceania and Africa which indicates that the disease is widely distributed, in tropical as well as in temperate areas of the world (Karmali and Fleming, 1979; Rettig, 1979).

There is no doubt that jejuni/coli GC are aetiological agents of diarrhoeic disease in human beings for the following facts (Blaser and Reller, 1981):

- 1) Patients with diarrhoea excret more campylobacters than healthy persons.
- 2) In outbreaks of gastrointestinal illness, campylobacters have been isolated from stools of affected persons but not from those who remained well and no other pathogen was found.
- 3) Campylobacters have been isolated simultaneously from stools and blood cultures from some patients with enteritis.
- 4) Convalescent patients have rising titres of serum antibodies.
- 4) Finally, ~~a~~ gastrointestinal illness was produced in volunteers who ingested cultures and campylobacters were isolated from their stools.

While the jejuni/coli GC are considered as frequent aetiological agents of human gastroenteric diseases, the isolation of C. fetus subsp. fetus has only been occasionally reported until now and it is thought that it is of little importance in human pathology. This is so because in the first works done in Brussels C. fetus subsp. fetus was found in only 3 out of 22,000 stool samples and in these 3 cases there was no association with diarrhoea (Butzler and Skirrow, 1979). Nevertheless, data from these first reports should be interpreted with caution because the bacteriological techniques employed could be the real cause of the low isolation rate of C. fetus subsp. fetus. In one of their first works Butzler, Dekeyser, Gossuin-Detrain and Dehaen (1973) placed the supernatants of centrifuged and filtered faeces onto Florent brilliant green agar and on a selective

thioglycollate agar and both media were incubated at 37°C; this filtration technique was followed until 1977 when these investigators gave up the search for C. fetus subsp. fetus mainly because of this initial lack of isolation (Lauwers, De Boeck and Butzler, 1978). Although media and temperature employed would have allowed the growth of C. fetus subsp. fetus it is possible that the filtration techniques used could have selected jejuni/coli GC from a mixed flora of campylobacters. An example of this is the work of Firehammer and Myers (1981) who using a similar technique with bovine faeces realized that colony counts of C. fetus subsp. fetus are more reduced by filtration techniques than counts of jejuni/coli GC and this happened to such an extent that they were unable to isolate any C. fetus subsp. fetus from calves, although the selective agar employed would have been able to support the growth of both campylobacters. From 1977 onwards, cephalothin was added to Butzler's culture medium and the incubation temperature used was 43°C (Lauwers, De Boeck and Butzler, 1978). C. fetus subsp. fetus would not therefore have been isolated either because of the inhibition of cephalothin (Karmali, De Grandis and Fleming, 1980) or the high incubation temperature used in most human bacteriological laboratories. Heinzer (1983) reported the isolation of 9 strains of C. fetus (C. fetus subsp. fetus) from faecal samples of patients with a clinical picture of febrile enteritis, entirely similar to an infection due to jejuni/coli GC. Recently, he recovered one strain from a diarrhoeic child (Heinzer, 1983, Personal Communication). He suggested that low isolation rates for C. fetus in most laboratories could be mainly due to inadequate bacteriological techniques for this

species. Nevertheless, Devlin and McIntyre (1983) inoculated 1,000 consecutive stools on Skirrow's medium incubated microaerophilically at 35°C for 48 hours and were unable to isolate C. fetus subsp. fetus while C. jejuni was frequently isolated, representing 41 per cent of all the bacterial enteropathogens recovered routinely in their laboratory. These investigators isolated two strains of C. fetus subsp. fetus from homosexual men suffering from diarrhoea. The analysis of 102 reported non-enteric human infections reveals that most patients had had one or more pre-existent major medical conditions, so that the C. fetus subsp. fetus in these cases was merely and opportunistic invader of weak patients (Rettig, 1979). The most common clinical manifestation is bacteremia without localized infection; recent reports indicate that most of the blood stream isolations are C. fetus subsp. fetus. As in ruminants, if the bacteremia occurs in a pregnant woman there is a risk of abortion or perinatal disease. The pathogenesis of systemic campylobacteriosis is not clear. Oral ingestion of ~~the~~ organisms similar to those in ovine infection has been suggested but not proved. Gastrointestinal infection or carriage as a predisposition to systemic disease has been postulated but not demonstrated so far, because only in exceptional cases has a positive stool culture been obtained from bacteremic patients (Rettig, 1979).

Marshall and Warren (1983) and Warren and Marshall (1983) described a campylobacter-like bacterium ("C. pyloridis") which grows in massive numbers beneath the mucus of the gastric antrum and its isolation is correlated with cases of gastritis and duodenal ulcer. The bacterium has up to four unipolar sheathed flagella,

grows microaerophilically and its taxonomic position is still uncertain. Recently, Fennell, Totten, Quinn, Holmes and Stamm (1983) isolated campylobacter-like organisms from rectal swabs of homosexual men with gastrointestinal symptoms. Phenotypically, these groups of microorganisms resemble C. jejuni but genotypically they are not related. It is suggested that they could be sexually transmitted.

In 1973 Véron and Chatelain described C. jejuni as "a rather poorly defined organism". They were able to examine only one strain because of the rarity of its isolation. Now, isolations of this species are so numerous that rapid advances have been made, especially in the methods of isolation and biochemical classification developed by medical sciences. This determined that the research for this thesis has been carried out in a way completely different to that in which it would have been done only a few years ago.

## 1) Campylobacter species in enteric disease of animals

### 1.1 Jejuni/coli GC

The great importance that this group of campylobacters has recently acquired in human medicine has notably influenced the development of research in veterinary science not only in contributing new bacteriological techniques but also orientating and encouraging numerous investigators in a search for reservoirs of human infection among animals and food products of animal origin. So, during the last few years, research on different species of domestic and wild animals has shown that these bacteria are more widespread among asymptomatic apparently healthy animals than was previously believed.

The campylobacters of this group are nowadays frequently isolated from a variety of domestic animals and this fact questions the validity of considering the mere isolation of them from a diarrhoeic animal as a possible cause of disease. These bacteria have been claimed to be the aetiological agents of winter dysentery in cattle, diarrhoea in calves, vibronic hepatitis in chickens, bluecomb disease (transmissible enteritis) in turkeys and dysentery in pigs. The role of these campylobacters as causative agent of enteric disease in all those animals is, at present, not well substantiated not only because their high prevalence among healthy carriers but also because attempts to produce enteritis by oral inoculation of pure cultures of campylobacters to conventional or gnotobiotic animals have generally met with limited success. Conventional animals were used in the only experiments in which production of disease has been achieved but doubt still remains because, in some cases, similar experiments with gnotobiotic animals of the same species have failed to produce the illness or, in others, different groups of investigators did not succeed in reproducing the disease even with conventional animals under similar field conditions. So, whether this group of campylobacters is able to produce a similar disease in animals as it was demonstrated for human beings, is still unknown. The problem is complex because jejuni/coli GC is composed by two species and a variety of serogroups and, the research work carried out during the last years shows that this group of bacteria is heterogeneous. On the other hand, it has been demonstrated that some strains of the jejuni/coli GC are frequently isolated as aetiological agents of abortions in sheep and it is known that



abortigenic strains are likely to cause septicemia in orally inoculated animals. This fact shows that there is a difference in virulence between apparently identical strains and the problem may lie in our lack of suitable laboratory tests to identify the possible enteropathogenic strains.

The enteric occurrence of the jejuni/coli GC in different animals will be considered here with a review on the research done on the possible role of this group as cause of enteric disorders in various domestic and laboratory animals, particular aspects of the relation with winter dysentery and the occurrence of these microorganisms in dairy cows will be also considered.

a) Jejuni/coli GC in ruminants

As has been described in the historical review, jejuni/coli GC like C. fetus subsp. fetus have long been known to cause abortion in sheep (Smibert, 1978; Firehammer, 1979; García, Eaglesome and Rigby, 1983). Recently, C. jejuni was also reported as a cause of abortion in goats (Anderson, Hamoud, Urbance, Rhoades and Bryner, 1983). Infection occurs by oral route and experimental exposure to the organism after 90 days of gestation may cause abortion (García, Eaglesome and Rigby, 1983). Nevertheless, apparently asymptomatic carriers are very common and healthy ruminants frequently harbour this group of microorganisms in their intestinal tracts to such an extent that some investigators considered it as part of the indigenous enteric flora (Smibert, 1965a; El Azhary, 1968). Smibert (1965a) found that 36 out of 214 (16.8 per cent) clinically normal sheep representing 6 of 8 flocks, excreted this group of campylobacters and he noted that in 14 to 20 month old sheep 24.8 per cent harboured



these bacteria but, in 2 to 10 years old sheep only 4.2 per cent yielded the microorganisms. El Azhary (1968) isolated jejuni/coli GC from 78 out of 339 (23 per cent) bovine faeces and from 10 out of 75 (13.3 per cent) ovine faeces taken at random. Clark and Monsborough (1979) isolated jejuni/coli GC from 30 out of 1,015 (2.95 per cent) ovine bile samples taken at random at a slaughter house.

Besides being commonly isolated from groups of healthy ruminants, other investigators have studied the possible correlation of these bacteria with diarrhoea of calves. Lederle (1963) isolated jejuni/coli GC from 8 out of 300 (2.7 per cent) duodenums of calves, some with enteritis. Firehammer and Myers (1981) cultured faecal samples from 127 diarrhoeic calves representing 25 herds and from only 3 healthy animals from within 3 of these herds; jejuni/coli GC was isolated from 51 (40 per cent) of the diarrhoeic calves and also from the three healthy controls. Prescott and Bruin-Mosch (1981) reported the isolation of this group of campylobacters from: 5 out of 202 (2.5 per cent) faecal samples of healthy cattle (calves from local farms and steers from slaughter house) and 3 out of 200 (1.5 per cent) diarrhoeic calves; 15 out of 110 (13.6 per cent) healthy sheep and only 1 out of 23 (4.3 per cent) diarrhoeic ovines; 2 out of 72 (2.7 per cent) healthy goats and 1 out of 27 (3.7 per cent) diarrhoeic caprines. Al-Mashat and Taylor (1980a) isolated jejuni/coli GC from the intestinal mucosa of 7 out of 47 (14.9 per cent) cattle with lesions that they considered similar to the ones described by Jones and Little (1931a and b) in natural cases of winter dysentery in cows and chronic enteritis in calves. These investigators (Al-Mashat and Taylor, 1980b) challenged three milk-fed and six conventional ruminant calves with oral inoculum of  $5 \times 10^{10}$  colony

forming units (CFU) of a jejuni/coli GC strain producing in all inoculated animals fever, diarrhoea and sporadic dysentery within one or three days PI. The animals were killed 10 to 16 days PI and presented thickening of the wall of the ileum, inflammation of the jejunum and ileum, mucoid ileal contents and enlargement of the mesenteric lymph nodes (MLN). Histologically, the lesions were restricted to the small intestinal mucosa and consisted of stunted villi, dilated crypts - some of which were filled with inflammatory cells - dilated capillaries and mononuclear cell infiltration. The bacterium was re-isolated from the ileum, caecum and colon of all inoculated animals and the jejunum, gallbladder and abomasum of some. No campylobacters were isolated from any enlarged MLN. The authors considered that the clinical and pathological syndrome produced in these experiments resembles the experimental cases described by Jones and Little (1931a and b).

Infection in adult cattle: Milk infection and association with faecal excretion

The fact that unpasteurized milk is considered one of the major vehicles of human campylobacter outbreaks (Taylor, Weinstein and Bryner, 1979; Porter and Reid, 1980; Robinson and Jones, 1981; Osterholm, Korlath, McCullough and Judy, 1982; Stalder, Isler, Stutz, Salfinger, Lauwers and Vischer, 1983; Pearson, Bartlett, Page, Jones, Lander, Lior and Jones, 1983) has initiated work on the role of the cow in the epidemiology of this infection and the possible routes by which contamination of milk occurs. One possible mechanism could be faecal contamination of the milk and to prove this the incidence and prevalence of jejuni/coli GC has been studied in

different dairy herds. A variable percentage of apparently healthy carrier cows has been reported: 0 per cent to 5.5 per cent (Oosterom, Engels, Peters and Pot, 1982), 0 to 10 per cent (Robinson, 1982), 27 per cent (Elegbe, 1983) and 64 per cent (Doyle and Roman, 1982). All these studies have been done with different methodologies but it is evident that as in many other animals a great variability exists. Robinson and Jones (1981) and Robinson (1982) took fortnightly rectal swabs from all members of two milking herds during 6 and 12 months respectively and found that:

- 1) jejuni/coli GC could be isolated from about 10 per cent of each herd during summer months, declining to zero during the winter and re-emerging during spring;
- 2) no symptoms were associated with the infection in any cow so this group of bacteria should be considered a commensal of cows;
- 3) several serotypes were detected in a herd at the same time and,
- 4) the excretion of campylobacters was low and intermittent.

For instance, Doyle and Roman (1982) isolated C. jejuni from rectal swabs of a grade "A" dairy herd with an incidence as high as 64 per cent at the end of autumn. Elegbe (1983) isolated jejuni/coli GC from the intestinal contents of 34 out of 127 (27.2 per cent) slaughtered cows (not specified as a dairy herd) with a level of  $10^6$  to  $10^{10}$  CFU/g faeces, while Oosterom, Engels, Peters and Pot (1982) found all 200 caecal contents investigated negative by direct plating and, in another survey, 11 out of 200 (5.5 per cent) were detected only if enrichment procedures were used showing that campylobacters were present in very small numbers. Older animals may harbour fewer campylobacters and this is another factor to consider when different

herds are compared (El Azhary, 1968).

Human milk-born outbreaks of campylobacteriosis might also be produced by direct excretion of bacteria through the milk. Up to now, only one case of natural campylobacter mastitis has been recorded (Anonymous, 1981). Intramammary inoculation of  $10^6$  jejuni/coli GC successfully produced experimental mastitis in which campylobacters were excreted before any clinical abnormality could be detected at levels of  $10^3$  to  $10^4$  CUF/ml milk and, in milder cases, low level excretion was demonstrated for up to 75 days (Lander and Gill, 1979 and 1980; Lander, 1980). Considering that relatively few campylobacters are needed to produce human disease - 500 organisms taken by mouth in 200 ml of milk - (Holt, 1981) undetected mild or sub-clinical mastitis could be a possible source of infection. Recently, two very sensitive methods have been developed to detect less than 1 organism per ml of milk and have showed that 1 to 2 per cent of farm bulk milk is contaminated (Doyle and Roman, 1982; Lovett, Francis and Hunt, 1983). Possibly, these new techniques could also be applied to detect individual low-excreter cows. Bacteremia in apparently clinically normal lactating cows might be a possible cause of subclinical mastitis. To test this, Bryner and Warner (1983) orally inoculated colostrum deprived and fed calves with  $10^9$  CFU C. jejuni and  $10^{10}$  CFU C. coli - isolated from aborted bovine and swine fetuses - and demonstrated that bacteremia occurs within 2 - 3 hours PI and lasts until 14th - 28th day PI.

Efficient pasteurization is effective in the prevention of milk-born campylobacter enteritis (Gill, Bates and Lander, 1981; Waterman, 1982) and all outbreaks occur in raw milk drinkers within

a large population drinking pasteurized milk (Robinson and Jones, 1981). As jejuni/coli GC is so common in cattle, pasteurization of milk is the only way to prevent human infection.

#### Winter dysentery and campylobacters

This disease has been known by other different names: winter scours, infectious diarrhoea, vibrionic enteritis, epizootic diarrhoea, black scours, epizootic enteritis and winter haemorrhagic enteritis. It is an acute, highly contagious disease of adult cattle characterized by a brief explosive attack of diarrhoea or dysentery. It occurs in housed cattle during the winter months. There is a little change in affected animals' temperature and pulse or respiratory frequency. Pyrexia, if present, often precedes the diarrhoea. High morbidity (3 to 100 per cent) and low mortality (1 - 2 per cent) are characteristics. The major economic loss is a moderate to severe decrease in milk production. Preceding the onset of diarrhoea it is common to find nasolacrimal discharge and cough, only exceptionally respiratory symptoms occur without ever presenting scours. Diarrhoea appears suddenly and is described as watery, foamy and the faeces often contain bubbles. Melena is often present (5 to 80 per cent) in scouring animals. Catarrhal inflammation of the small intestine - particularly the jejunum and upper ileum - and only mild changes in the colon, caecum and rectum have been described. Congestion, swelling, petechiation and/or serofibrinous exudation of the nasal cavity, trachea or larger bronchi, sometimes parietal pleuritis or ecchymosis of the diaphragm have been reported. Recovery usually occurs (Campbell and Cookingham, 1978).

After the work of Jones and Little (1931a) jejuni/coli GC were

considered by many investigators as the aetiological agent of winter dysentery until MacPherson (1957) disputed these conclusions and proposed that the disease is caused by a virus and bacteria are only secondary invaders. He was able to reproduce the disease by subcutaneous inoculation of 5 cc of 1/10 diluted and filtered faeces from sick animals, into two 18-month -old steers. At the fourth day PI an increase in nasolacrimal secretion was observed, rectal temperature was raised and the leucocyte count dropped. Coincident with the development of these symptoms diarrhoea was detected and by the 7th day PI frank dysentery was apparent. After this short illness the animals recovered but close examination of the faeces showed that flecks of blood persisted for a period of 14 to 21 days. Furthermore, filtered faeces taken from the experimentally diseased animals regularly, reproduce the disease. Although the virus was not isolated MacPherson's (1957) work generated considerable doubt on the role of campylobacters in the aetiology of this condition and two years later, Komarov, Goldsmith, Kalmar, Adler and Edyed (1959) reproduced the disease in bovines with virus propagated in chick embryos or mice. Later, Charton, Faye, Lecoanet, Desbrosse and Le Layec (1963) recovered an enterovirus from scouring bovines, were able to maintain it in tissue cultures and also reproduced the disease in cattle. Scott, Kahrs, Campbell and Hillman (1973) attempted to isolate C. jejuni from approximately twenty herd outbreaks of winter dysentery and although rigorous microbiologic techniques were applied, all the cases were consistently negative. Other authors have isolated coronavirus from diarrhoeic adult bovines and recently a new viral particle provisionally named the "small round virus" ("petit virus

rond") has been frequently observed since 1981 in bovines suffering from winter dysentery by researchers of the Laboratoire National de Pathologie Bovine in France (Brugere-Picoux and Vialard, 1983). Until now, many other investigators have tried to discover the elusive aetiology of this disease without any results and while some findings are published many are not, mainly because of inconsistent or negative results (Campbell and Cookingham, 1978). It is evident that the disease as it is generally described has symptoms and lesions affecting the respiratory system which have never been described in campylobacteriosis and, the diarrhoea is severe, appearing suddenly with blood not only as flecks but also as whole blood, characteristics which differ from the natural or experimental cases of diarrhoea due to campylobacters. Nowadays, it is generally believed that the disease is probably caused by a virus although a defined specific aetiological agent is not known yet (Campbell and Cookingham, 1978).

b) Jejuni/coli GC in pigs

Since Vibrio coli was reported as a possible agent of swine dysentery (Doyle, 1944 and 1948; James and Doyle, 1947) only few investigators have succeeded in producing a similar disease by oral administration of pure cultures of campylobacters. In a small number of conventional pigs some of the inoculated animals developed dysentery and others transient diarrhoea or remained healthy (Roberts, 1956a; Truszczyński, 1957). The description of the inoculated vibrios was similar to that of Vibrio coli (jejuni/coli GC HT) (Roberts, 1956b; Truszczyński, 1957). Truszczyński (1957) was able to reproduce the typical disease in only a few of the orally inoculated conventional pigs and the same investigator found V. suis subsp. coli (jejuni/coli





GC HT) in 12 out of 20 healthy pigs. Deas (1960) failed to produce disease either by oral inoculation of vibrios of type II (jejuni/coli GC HT) or type I (C. fetus subsp. fetus or "C. hyointestinalis" HT) to 8 weeks old conventional pigs. Davis (1961a and b) found that only one culture out of 18 appeared to be able to induce swine diarrhoea and five attempts to produce disease involving 23 pigs were unsuccessful; in addition, when the diarrhoeic disease was produced in other experiments it had been a mild disease, not typical of the dysentery originally described by Doyle (1944). Experiments in gnotobiotic piglets failed to produce clinical or pathological signs of dysentery although V. coli (jejuni/coli GC) was detected, persisted in the intestine and was excreted in high numbers ( $10^9$  x g/faeces) (Andress, Barnum and Thomson, 1968; Kashiwazaki, Namioka and Yabiki, 1971). No gross abnormalities were observed and only the lamina propria of infected pigs was markedly more cellular (Andress, Barnum and Thomson, 1968). By immuno-fluorescent staining vibrios were never observed in the epithelium or lamina propria, only individual organisms were scattered through the MLN (Kashiwazaki, Namioka and Yabiki, 1971). In other experiments, conventional pigs were fed with cultures of V. coli or minced intestine from gnotobiotic pigs heavily infected with the bacterium and, although the organism was isolated from all the pigs no clinical signs of disease were observed (Andress and Barnum, 1968). These results persuaded many workers that campylobacters are not the cause of swine dysentery and this concept was further supported by the reproduction of the disease by oral inoculation of pigs with cultures of an anaerobic spirochaete (Taylor and



Alexander, 1971) called Treponema hyosdysenteriae (Taylor, 1981). Later, the pathogenicity of campylobacters for pigs was re-examined and after oral inoculation of gnotobiotic piglets with human strains of C. jejuni no clinical signs appeared and only a mild increase in the fluidity of intestinal contents was detected, in the absence of any histopathological changes (Prescott, Manninen and Barker, 1982). More recently, Olubunmi and Taylor (1982) were able to produce, by oral inoculation of C. coli, a mild colitis in hysterectomy derived, colostrum deprived piglets and, to a lesser extent, in conventional sucking piglets but failed to detect clinical signs in weaned pigs. The disease consisted in symptoms of depression, fever and diarrhoea or soft faeces containing mucus which appeared between 2 to 5 days PI and, histologically mild villous atrophy and inflammation of the small intestine. These investigators (Taylor and Olubunmi, 1981) considered that natural cases from which C. coli had been isolated had lesions which resembled the experimentally produced disease.

On the other hand, jejuni/coli GC are very frequently isolated from the intestinal tract of swine. Smibert (1978) considered this group of campylobacters to be normal in the intestinal faecal flora of young pigs and suggested that the large numbers of bacteria seen in the faeces of pigs with swine dysentery may be due to their secondary involvement in the infection or that they may just grow well in the intestinal environment caused by the disease. Jejuni/coli GC were isolated from 2 out of 208 (0.96 per cent) faeces of healthy animals (Prescott and Bruin-Mosch, 1981) and 4 out of 170 (2.4 per cent) of diarrhoeic animals, 37 out of 80 (46.2 per cent) faeces

(El Azhary, 1968), 182 out of 300 (60.7 per cent) intestinal contents (Oosterom, 1980), 103 out of 173 (59.5 per cent) faeces (Sticht-Groh, 1982) and 29 out of 50 (58 per cent) samples of bile from healthy slaughtered pigs (Rosef, 1981a). Furthermore, von G6rgen, Kirpal and Bisping (1983) compared the rate of isolation from normal and diarrhoeic pigs finding that Campylobacter spp. were isolated from 161 out of 200 (80 per cent) faeces and 46 out of 48 (95.8 per cent) intestines from normal animals versus only 49 out of 74 (66.2 per cent) faeces and 22 out of 40 (55 per cent) intestines of diarrhoeic animals. These investigators also found that the numbers of campylobacters per gram of faeces ranged between  $10^2$  and  $10^5$  in both healthy and diseased pigs and they also demonstrated the same stable concentration in the faeces of two healthy animals over a period of 2 months. Even more, after oral inoculation of young pigs with Campylobacter sp. or Treponema sp. or both together, no difference in the excretion rate of campylobacters was noted. They concluded that as campylobacters can be found with a percentage of 48 to 100 per cent in the intestine of pigs - according to different groups of age and weight - they should be considered as part of the normal flora of pigs.

c) Jejuni/coli GC and C. laridis in poultry

In the past, jejuni/coli GC have been incriminated as the aetiological agents of vibronic hepatitis (Hofstad, 1956) and it was even also recommended that an examination of a stained cecal smear for vibrios would be a useful aid in the diagnosis of the disease (Truscott and Stockdale, 1966). Nowadays, the disease is rarely diagnosed (García, Eaglesome and Rigby, 1983) and it is

appreciated that many chickens harbour these campylobacters.

Different studies have proved that a high percentage of chickens are apparently healthy carriers: 91 per cent (Ribeiro, 1978), 83 per cent (Grant, Richardson and Bokkenheuser, 1980), 82 per cent (Shanker, Rosenfield, Davey and Sorrell, 1982), 72 per cent (Simmons and Gibbs, 1979), 47 per cent (El Azhary, 1968), 30 per cent (Goren and Jong, 1980) and 24 per cent (Prescott and Bruin-Mosch, 1981). This last percentage was low because only one of three groups of chickens investigated harboured campylobacters showing that some flocks are naturally free from infection. Furthermore, Goren and Jong (1980), Cruickshank, Egglestone, Gawler and Lanning (1982) and Rosef and Kapperud (1982) described that only 41.6 per cent, 21 per cent and 10 per cent, respectively, of the flocks studied were infected. Recently, Shanker, Lee and Sorrell (1983) compared farms of two districts, one with high and the other with low incidence of infection, and concluded that good management and hygienic conditions can prevent the infection. Naturally infected chickens have between  $10^4$  to  $10^7$  CFU per gram of faeces (Grant, Richardson and Bokkenheuser, 1980; Smeltzer, 1981; Oosterom, Notermans, Karman and Engels, 1983; Wempe, Genigeorgis, Farver and Yusufu, 1983).

Previously, bluecomb disease of turkeys has been associated with this group of campylobacters (Truscott, Connell, Ferguson and Wills, 1960; Truscott and Morin, 1964) but later on, it was determined that the disease is produced by virus with or without secondary bacterial invaders (Wooley and Gratzek, 1969). On the other hand, jejuni/coli GC have been isolated from 100 per cent of 600 caecal contents (Luechtefeld and Wang, 1981) and  $10^4$  to  $10^7$  CFU per gram were detected

(Luechtefeld, Wang, Blaser and Reller, 1981). Nevertheless, as in chickens it is possible to raise turkey poults free from campylobacter infection (Acuff, Vanderzant, Gardner and Golan, 1983).

Many other apparently healthy wild birds harbour jejuni/coli GC as pigeons, sparrows, starlings, crows, ravens, blackbirds, ducks, gulls, puffins, etc. (Smibert, 1969; Luechtefeld, Blaser, Reller and Wang, 1980; Luechtefeld, Cambre and Wang, 1981; Prescott and Bruin-Mosch, 1981; Weber, Lembke and Ketter, 1981; Rosef, 1981b; Fenlon, Reid and Porter, 1982; Kuhn, Whitla and Montali, 1983; Kapperud and Rosef, 1983). Kapperud, Rosef, Rostad and Lid (1983) however related a high rate of isolation of C. laridis with puffin chick mortality. Campylobacters faecal counts range between  $10^5$  to  $10^6$  in wild birds (Fenlon, Reid and Porter, 1982).

Most chicken isolates have been found to be C. jejuni with only a small proportion of C. coli or C. laridis (Rosef and Kapperud, 1982; Smeltzer, 1981). C. jejuni biotype 2 is likely to be associated with poultry (Skirrow, Fido and Jones, 1981; Cruickshank, Egglestone, Gawler and Lanning, 1982). C. laridis is only frequently isolated from gulls (Benjamin, Leaper, Owen and Skirrow, 1983). C. jejuni biotype 1 has been isolated from turkeys (Acuff, Vanderzant, Gardner and Golan, 1983). C. jejuni and C. coli can be isolated together from the intestinal tract of the same chicken (Harvey and Greenwood, 1983).

It has been shown that poultry and birds are, in general, natural asymptomatic carriers of jejuni/coli GC and they seem to be quite resistant to enteric disease due to these bacteria. Nevertheless, diarrhoea was induced under experimental conditions in more than

80 per cent of 25 three-day -old chickens by oral inoculation of  $10^8$  campylobacters (Escamilla, Torres and Ruiz-Palacios, 1981; Ruiz-Palacios, Escamilla and Torres, 1981). In order to test the pathogenicity of human campylobacters Sanyal, Neogi, Islam and Huq (1983) utilized this model and measuring the volume of fluid in the gut they determined a peak effect (81 per cent of the chicks) at the 5th day PI. Nevertheless, inoculation of 25 chickens with  $10^8$  campylobacters produced colonization but not intestinal invasion of the gut or illness (Butzler and Skirrow, 1979). Similarly, none of fourteen isolates of C. jejuni or one of C. coli from various human and animal sources produced diarrhoea or death in 3-day -old chickens inoculated orally and observed during three days (Manninen, Prescott and Dohoo, 1982) despite the use of the same method of Ruiz-Palacios, Escamilla and Torres (1981). The native microflora of the gut could influence the outcome as it has been shown in experimental studies with monoxenic (monocontaminated) and holoxenic (conventional) chickens that previous colonization by the normal flora sharply reduced the colonization of campylobacters (Soerjadi, Snoeyenbos and Weinack, 1982). The gut microflora is not the only cause of resistance to infection as in other experiments it was demonstrated that inoculation of gnotobiotic chickens with C. jejuni failed to produce diarrhoea (Manninen, Prescott and Dohoo, 1982). Perhaps the cause of these controversial results could be the different pathogenicity of similar strains which we do not know how to differentiate.

The chicken embryo has also been used as a model to study the pathogenicity of human strains of jejuni/coli GC. Inoculation of

2 strains into the chorioallantoic membrane of 11 and 17-day-old embryos was performed and the recovery of bacteria from the heart and liver was measured after 24 and 48 hours of incubation. It was seen that older embryos are more resistant to infection than younger ones and the two strains of campylobacter tested displayed a different ability to invade the chorioallantoic membrane (Davison and Solomon, 1981).

d) Jejuni/coli GC in dogs and cats

The carriage of jejuni/coli GC by dogs has been frequently described. Some authors cited a higher rate of isolation from diarrhoeic dogs and cats than from non-diarrhoeic ones (Vandenberghe, Lauwers, Plehier and Hoorens, 1982; McOrist and Browning, 1982) while others described the same frequency of isolation for symptomatic or asymptomatic animals (Hosie, Nicolson and Henderson, 1979; Bruce, Zochowski and Fleming, 1980; Bruce and Fleming, 1983; Simpson and Burnie, 1983). The prevalence is higher in stray dogs than in dogs of known ownership (Simpson and Burnie, 1983). In kennels there is also a higher rate of isolation and many dogs might acquire the infection there (Burnie, Simpson, Lindsay and Miles, 1983). Probably, not more than 5 per cent of human campylobacter enteritis cases in Britain are associated with dogs and cats; many recorded cases involve very young children in close contact with puppies with diarrhoea (Skirrow, 1981). A human case of enteritis due to C. jejuni was reported in which the infection was probably caused by a healthy cat which excreted  $3.7 \times 10^7$  C. jejuni per gram of faeces. Interestingly, when the cat was killed C. jejuni was isolated from the caecum and rectum but not from other intestinal

sites or organs (Blaser, Weiss and Barrett, 1982).

Attempts to produce disease with  $10^{10}$  organisms of two human C. fetus subsp. jejuni strains (jejuni/coli GC) in 6-week -old conventional kittens and 7-week -old conventional puppies have failed. Although the bacteria were excreted from the second or third day PI only transient diarrhoea was produced showing that these animals were not susceptible to the disease but became healthy carriers (Prescott and Karmali, 1978). The inoculation of gnotobiotic puppies with  $10^{10}$  of a human and a canine strain of C. jejuni induced mild diarrhoea and tenesmus during a period of 36 - 72 hours PI. Lesions were limited to typhilitis and colitis. Congestion of colonic mucosa, associated loss of goblet cells, attenuation of the surface epithelium, hypertrophy of glands and neutrophil inflammation were observed. C. jejuni was established at over  $10^{10}$  organisms per gram of colonic contents but did not invade the mucosa (Prescott, Barker, Manninen and Miniats, 1981).

#### e) Jejuni/coli GC in rodents

The incidence of jejuni/coli has been studied in laboratory and some pet rodents. Different pet stores have an incidence of 40 to 80 per cent of their hamsters excreting C. jejuni in their faeces (Fox, Hering, Ackerman and Taylor, 1983). The excretion rate for different populations of laboratory hamsters varies between 0 to 100 per cent (Fox, Zanotti and Jordan, 1981; Ackerman, Newcomer and Fox, 1982). Interestingly, some jejuni/coli GC strains isolated from hamsters seem to be different because they are very difficult to subculture and do not grow in broth medium (Fox, Zanotti and Jordan, 1981). Over a twelve months period, 168 ferrets from two



commercial breeders were examined and 61 per cent of the animals excreted jejuni/coli GC (Fox, Ackerman and Newcomer, 1983). A negative or low percentage of isolation was found in laboratory guinea pigs, rabbits, rats and mice (Weber, Lembke and Schäfer, 1982). Blaser, Duncan, Warren and Wang (1983) found that adult laboratory mice (strain HA-ICR) do not carry C. jejuni and that this group of campylobacters are not bowel commensals in this strain. In general, the isolation of jejuni/coli GC from rodents is not associated with disease but nevertheless, an outbreak of C. jejuni <sup>carnivorous</sup> abortions in mink, associated with the development of mucoid droppings occasionally tinged with blood was described (Hunter, Pettit and Prescott, 1983).

Laboratory rodents have proved to be very useful for pathogenic studies with human isolates. Intragastric feeding of  $10^8$  jejuni/coli GC human strains to neonatal (Field, Underwood, Pope and Berry, 1981) or adult mice (Blaser, Duncan, Warren and Wang, 1983) produced 100 per cent of infection. In the work with neonatal mice it was found that the greatest number of microorganisms was recovered from the caecum and large intestine ( $10^7$ ) while the small intestine had lower numbers (between  $10^2$  and  $10^3$ ) and the stomach was not consistently colonized. Mortality varied between 0 to 13 per cent according to the campylobacter strain used. Scanning EM photographs showed numerous campylobacters in and below the ~~are~~ mucus gel which covers the villi of the ileum in neonatal mice and the organisms could be isolated from faeces for 2 to 3 weeks PI (Field, Underwood, Pope and Berry, 1981). In adult mice transient bacteremia is a consistent finding observed in nearly 100 per cent

of inoculated animals within 10 minutes PI but decreased to 40 per cent within 1 to 6 hours and to 20 per cent within 12 to 24 hours. Interestingly, the pattern of colonization of adult mice was completely different from that of neonatal animals, in this case, the number of C. jejuni was higher in the small intestine ( $10^3$  to  $10^4$ ) than in the caecum and colon ( $10^2$  to  $10^3$ ). Infected mice showed no signs of illness and only mild inflammatory changes were found in the small intestine but not in the large bowel. Persistent excretion was the rule until 14 months PI but chronic histological changes were not consistently detected (Blaser, Duncan, Warren and Wang, 1983). A difference was also detected in the physiological changes which occurred in campylobacter enteritis of young mice - 4 to 6 weeks old - and old mice - 24 to 26 weeks old - inoculated with jejuni/coli GC. Young inoculated mice showed significantly lowered D-glucose and D-galactose absorption in the distal four-fifths of the small intestine while in old inoculated mice the absorption of these solutes remained unaltered (Madge, 1980). The cause of these differences is not known but perhaps the intestinal flora could be involved because it has been demonstrated that microbial antagonism within the lumen of the gut is one of the possible mechanisms of resistance to colonization of campylobacters. When gnotobiotic (germ-free) mice are sequentially infected, first with human faecal flora and then with strains of C. jejuni, the campylobacters are rapidly eliminated. If faeces from a patient with acute campylobacteriosis are inoculated to germ-free mice the animals shed C. jejuni at a concentration of  $10^8$  CFU/g faeces during several weeks without showing any clinical signs. When only

C. jejuni is given to the mice it readily colonizes but if faecal microflora of a healthy volunteer is inoculated the campylobacter is eliminated within a week after the addition of the flora (Andreumont, Leonard, Goldstein, Pean, Pequet and Tancrede, 1983).

Culture supernatants of jejuni/coli GC strains induce a net sodium secretory response in jejunal segments of adult rats suggesting that this group of campylobacters produces one or more substances that might be involved in the mechanism of diarrhoea (Fernández, Fagundes Neto, Fernandes, Almeida Pedra and Trabulsi, 1983). Employing a mouse uterus infection model to test human C. jejuni strains it was demonstrated that some strains are invasive, others produce fluid accumulation in the uterus while others cause both effects. These two pathogenic mechanisms might be involved in the production of campylobacter enteritis (Kita, Katsui, Yanagase and Kashiba, 1983).

Neonatal rats and rabbits were similarly colonized as neonatal mice - therefore the highest numbers of campylobacters were in the large intestine and caecum - whereas neonatal hamsters were resistant to colonization (Field, Underwood, Pope and Berry, 1981). Fox, Zanotti and Jordan (1982) were able to orally infect hamsters with a jejuni/coli GC strain which was streptomycin resistant and the inoculated bacterium was recovered from the large and small intestine up to 16 weeks PI. Enteritis was only produced in a group of animals fed with a high sucrose diet but not in normally fed hamsters.

#### f) Jejuni/coli GC in other animals

Jejuni/coli GC can also be isolated from a variety of zoo animals including primates, felids, hoofed animals, birds and even reptiles (Luechtefeld, Cambre and Wang, 1981). These authors found

that faecal samples from diarrhoeic zoo animals had a higher percentage (31.8 per cent) of this group of campylobacters than non-diarrhoeic (5.6 per cent), although the authors considered that these results were difficult to interpret because in most cases other enteric pathogens were detected together with the campylobacters.

Jejuni/coli GC has been isolated from a large proportion of imported simian primates with diarrhoea. The disease did not cause mortality. One of the carrier monkeys (Maccaca fascicularis) was killed and jejuni/coli GC was isolated from duodenum, ileum, caecum, colon, rectum, peritoneum, stomach and, interestingly, a heavy pure growth was obtained from the gall and urinary bladder (Tribe, Mackenzie and Fleming, 1979; Tribe and Frank, 1980). Reproduction of the disease in non-human primates has met with variable results. While most investigators failed to produce the disease, others succeeded in inducing a mild disease with inappetence and diarrhoea of short duration and in these cases bacteremia was present for only 2 to 3 days PI; later, the organisms could be recovered from liver and gallbladder. Only exceptionally was severe diarrhoea produced (Fox, 1982).

### 1.2 Campylobacter fetus subsp. fetus

The economic importance of enzootic abortion in sheep and sporadic abortion in cattle due to these subspecies have stimulated numerous studies on the incidence of these microorganisms in the reproductive and digestive tract of bovines and ovines. Florent (1959a and b) demonstrated the presence of Vibrio fetus intestinalis (C. fetus subsp. fetus) in the faeces of cattle from herds in which sporadic abortion was occurring and he was able to experimentally

reproduce the disease by i/v inoculation of the faecal isolates. Besides, by means of brilliant green agar as a selective medium Florent (1959b) also frequently found V. fetus intestinalis (probably, C. fetus subsp. fetus or CHI HT) in the digestive tract of apparent healthy bovines, ovines and porcines and arrived at the conclusion that this bacterium should be considered as a normal saprophitic microorganism of the intestines of these animals. In this work, although only few animals were investigated the frequency of isolation was higher from calves (67 per cent) than from cows (7 per cent). Later on, this finding was confirmed by El Azhary (1968) who - by means of a combination of brilliant green selective medium and filtration techniques - and working with faecal samples taken at random isolated V. fetus intestinalis (probably, C. fetus subsp. fetus or CHI HT) from 76 out of 339 bovines (22.4 per cent), 8 out of 75 ovines (10.6 per cent), 10 out of 80 porcines (12.5 per cent) and 2 out of 60 poultry (3.3 per cent). Furthermore, he demonstrated that the incidence of this microorganisms in the faeces of cattle with problems of sporadic vibrionic abortion was the same as that of those without a history of the disease. He isolated V. fetus intestinalis from approximately 60 per cent of 2 months old calves and 10 per cent of adult cows and speculated that older cattle possibly developed more immunity resulting in a smaller population of vibrios in the intestinal tract. Clark, Monsborough and Dufty (1969) isolated V. fetus intestinalis (C. fetus subsp. fetus and jejuni/coli GC HT) from the faeces of 174 out of 220 heifers (79 per cent). Mehle (1971) isolated 110 vibrios from 407 cows in 7 farms (29.2 per cent) and classified the strains by the

Mitscherlich complement fixation test finding that this test classified 15 per cent of the isolated strains as V. fetus type 1 and 9 per cent as V. fetus type 2 and found that the Mitscherlich test was not useful in distinguishing the strains which cause enzootic infertility in cattle. He failed to reproduce sterility with his V. fetus type 1 isolates.

The natural infection of the bovine and ovine gallbladder is not a particularly unusual event (Firehammer, Lovelace and Hawkins, 1962; Bryner, O'Berry, Estes and Foley, 1972). Campylobacters (C. fetus subsp. fetus, CHI or "C. fecalis" HT) were isolated from 88 out of 525 (17 per cent) cattle selected at random when slaughtered and from 29 out of 186 (16 per cent) sheep from 9 flocks (Bryner, O'Berry, Estes and Foley, 1972). Intravenous inoculation of pregnant sheep with bile isolates of C. fetus subsp. fetus consistently produced abortion but intraruminal or oral inoculation failed or gave lower rate of abortion (Firehammer and Hawkins, 1964; Bryner, O'Berry, Estes and Foley, 1972). Parenteral inoculation of cattle, sheep, rabbits, guinea pigs and mice showed that C. fetus subsp. fetus had a hepatic tropism infecting the gallbladder with subsequent colonization of the intestine via the common bile duct (Bryner, Estes<sup>Foley</sup> and O'Berry, 1971).

The organism can also occur in the intestinal tract of cows (Watson, Hunter and Bellhouse, 1967), magpies (Meinershagen, Waldhalm, Frank and Scrivner, 1965; Waldhalm, Mason, Meinershagen and Scrivner, 1964) and ravens (Dennis, 1967a) which probably serve as secondary reservoir of infection of ruminants. Ravens became infected by oral inoculation of contaminated sheep placenta or cultures of V. fetus intestinalis (the strains could be considered to be

either C. fetus subsp. fetus or jejuni/coli GC) and developed mucoid diarrhoea for the first five days; inversely, oral inoculation of pregnant ewes with the experimentally infected raven faeces produced the birth of weakly infected lambs (Dennis, 1967a). Herring and black-backed gulls have also an unclassified campylobacter which may be C. fetus subsp. fetus or CHI (HT) (Fenlon, Reid and Porter, 1982).

Other investigators related the isolation of C. fetus subsp. fetus to the lesions of the intestinal tract where this bacterium was isolated. Lederle (1963) isolated 27 strains of Vibrio spp. from the duodenum of 300 (7 per cent) calves, of which 224 had some degree of inflammation; serologically, 13 strains were considered identical to V. fetus (C. fetus subsp. fetus HT). Allsup, Matthews, Hogg and Hunter (1972) isolated V. fetus var. intestinalis (Campylobacter spp. HT) from the intestinal contents of a ten-day-old calf with lesions of haemorrhagic enteritis and from 7 out of 8 calves of the same herd - 3 of them suffering from dysentery. Allsup and Hunter (1973) isolated vibrios (Campylobacter spp.) from the alimentary tracts of 36 out of 258 (14 per cent) diseased calves and 15 out of 237 (6.3 per cent) healthy calves and, from the faeces of 40 out of 130 (30 per cent) diseased and 15 out of 90 (16.7 per cent) healthy calves. These investigators found that the number of campylobacter positive animals was significantly greater in the diseased than in the healthy group of calves ( $P < 0.1$ ) and they also recovered more scanty cultures from healthy than from diseased calves. In this work, the total percentage of isolations of Campylobacter spp. was much lower than the obtained by El Azhary (1968) in bovine faeces taken at random (45.5 per cent)



and this could be due to the different bacteriological techniques employed by Allsup and Hunter (1973) who used sheep blood agar and serum dextrose agar with inhibitors and filtration procedures. Allsup and Hunter (1973) serologically tested 25 strains and found that 15 were related to V. fetus intestinalis (C. fetus subsp. fetus HT). Allsup, Matthews, Hogg and Hunter (1972) orally inoculated two milk-fed calves with vibrios (Campylobacter spp.) isolated from a calf which had died of enteritis: slight pyrexia and intermittent scouring were recorded and the organisms were excreted in the faeces for six weeks and were isolated from the jejunum and MLN of both calves.

Al-Mashat and Taylor (1980a) isolated 8 strains of C. fetus subsp. intestinalis (C. fetus subsp. fetus) from 30 calves (26.6 per cent) and none from 17 adult bovines one year old or more. In one case they reported the simultaneous isolation of C. fetus subsp. jejuni (jejuni/coli GC) and C. fetus subsp. fetus from the same animal. They described lesions in the small intestine similar to those previously reported in natural cases of winter dysentery and enteritis in calves (Jones and Little, 1931a and b) characterized by stunting of the villi, capillary dilation and presence of large numbers of plasma cells, mononuclears and eosinophils within the mucosa. The authors suggested that C. fetus subsp. fetus might be associated with these intestinal lesions. To demonstrate this, Al-Mashat and Taylor (1983b), in two separate experiments with conventional animals, orally inoculated with  $10^9$  C. fetus subsp. intestinalis (C. fetus subsp. fetus) three milk-fed calves and three ruminant calves. All inoculated calves presented fever and diarrhoea

with mucus containing occasional spots of blood. C. fetus subsp. fetus was recovered from the faeces, small and large intestine and, less frequently, from the MLN, liver and gallbladder. Five controls were used and they were free from C. fetus subsp. fetus. The lesions were found in the small intestine especially in the ileum and, to a lesser extent, in the jejunum and abomasum: macroscopically the mucosa was thickened and slightly congested and microscopically the ileal mucosa had stunted villi, disruption and metaplasia of luminal epithelial surface and crypts filled with inflammatory cells. The MLN were pale and enlarged. The authors considered that C. fetus subsp. fetus was the causal agent of the syndrome described. Nevertheless, it should be pointed out that other superimposed intestinal pathogens were detected in inoculated and control animals during the experiment, such as coccidiosis and also other diseases occurred because respiratory problems were detected.

### 1.3 "Campylobacter fecalis"

Firehammer (1965) described "faecal type vibrios" ("C. fecalis") which are very similar to C. sputorum subsp. bubulus but differed by the consistent production of large amounts of catalase. This microorganism was isolated from sheep faeces and the catalase negative variety may also be found in the bovine semen and vagina (Smibert, 1974). A "C. fecalis-like" similar in all respects but differing in the small size of the cell width (0.2  $\mu$ ) was isolated from the caecum and colon of mice. The bacterium forms part of the flora which colonizes the mucus layer of healthy mice (Roach and Tannock, 1979).

"C. fecalis" was also described associated with the bacterial

flora of bovine pododermatitis (Cornelisse, van Asten, Peterse and Toussant-Raven, 1982).

Firehammer (1979) considered that there is no evidence that the microorganism is pathogenic for man or animals. Later on, Al-Mashat and Taylor (1980a) isolated a campylobacter strain from lesions of the gastrointestinal tract of cattle provisionally classifying it as "C. fecalis". The designation "C. fecalis" was uncertain and the strain was sent to Skirrow who considered that it might be an unusual form of C. fetus subsp. fetus (Al-Mashat and Taylor, 1980a). "C. fecalis" was isolated from an abomasal lesion of an 18 month-old heifer and this strain was used to infect calves in two experiments in which 3 milk-fed and 3 ruminant calves were orally infected with approximately  $10^{10}$  bacteria (Al-Mashat and Taylor, 1981). Diarrhoea was not produced and only mild changes in faecal consistency or blood and mucus were noted. Mild abomasitis and ileitis were described in all 6 infected bovines. "C. fecalis" was re-isolated from the intestines and gallbladders of all infected animals and from the abomasum and livers of some. Although the MLN were enlarged no campylobacters were isolated from them. The authors suggested that this bacterium should be regarded as a primary pathogen of cattle able to initiate the clinical and pathological alterations mentioned above.

#### 1.4 Campylobacter sputorum subspecies bubulus

This species is found in the genital tract of male and female cattle and sheep and can be isolated from semen, preputial and vaginal mucus of normal animals (Smibert, 1974). Florent (1959b) also described the isolation of Vibrio bubulus (C. sputorum subsp. bubulus)

from bovine faeces. Lederle (1963) cited the isolation from the duodenum of calves of six catalase negative strains, four with the characteristics of V. bubulus and the other two were different because they were not able to grow in 3.5 per cent sodium chloride. Mehle (1971) isolated 13 strains classified as V. bubulus from the intestinal tract of cows.

## 2) Campylobacters and proliferative enteropathies

Vibrioid bacteria have also been described associated with various proliferative lesions of the intestinal tract of many species but the isolation of the presumably involved campylobacters has only been achieved in some of the diseases studied and in no case has it been possible to reproduce the typical pathology with the oral inoculation of the isolated campylobacters. One of the animal species in which this type of disease has been studied in detail during recent years is the pig. C. sputorum subsp. mucosalis (C. mucosalis) is associated with porcine intestinal adenomatosis, necrotic enteritis, regional ileitis and proliferative haemorrhagic enteropathy. The normal habitat of the microorganism is the oral cavity and it is not present in the intestinal tract of healthy pigs. In intestinal adenomatosis, however, C. mucosalis can be recovered from the diseased mucosa of the small intestines in large numbers and is often strictly confined to the site of the lesion. The ultrastructure of adenomatous epithelial cells resembles that of undifferentiated crypt cells. These immature enterocytes carry intracellular curved bacteria laying free in the apical cytoplasm and often surrounded

by a small unstained halo (Rowland and Lawson, 1974; Lawson, 1982). Intracellular vibrioid bacteria are a constant feature of the disease but oral inoculation of C. mucosalis or adenomatous mucosa to conventional pigs of different immunological status or gnotobiotic piglets have consistently failed to reproduce the proliferative disease. C. mucosalis colonizes the intestine of conventional pigs poorly or gnotobiotics heavily without invasion of enterocytes (Roberts, 1978; Roberts, Lawson and Rowland, 1980a and b; McCartney, 1982). Dual combined infections of C. mucosalis with rotavirus, Cryptosporidium sp. or ETEC did not produce adenomatosis. In contrast, in tissue cultures, C. mucosalis attaches specifically to a number of cell lines and when attachment takes place intracellular multiplication occurs and may persist for long periods (Rajasekhar, 1981).

In the first bacteriological studies of porcine intestinal adenomatosis Lawson and Rowland (1974) considered that the disease was correlated with the presence of C. mucosalis which was found only in cases of porcine intestinal adenomatosis whilst other catalase positive and serologically distinct campylobacters were isolated either from normal or from diseased animals and were not considered relevant to this disease and were generically named as C. coli. Later on, a bacteriological study was presented in which strains of C. coli type I were differentiated by the production of  $H_2S$  in insensitive media (TSI) (Lawson, Rowland and Wooding, 1975). Some years later, Gebhart, Ward, Chang and Kurtz (1983) proposed to designate all pig strains similar to C. fetus subsp. fetus but which only differ in the production of  $H_2S$  in TSI as "C. hyointestinalis"

(CHI). They isolated CHI alone or together with C. mucosalis from the majority of naturally occurring cases of swine proliferative ileitis that they had studied. By fluorescent antibody tests they found that CHI was present within epithelial cells of the ileal crypts and suggested that CHI was involved as a possible agent. Nevertheless, oral inoculation of swine with pure cultures of CHI or C. mucosalis separately or both together failed to reproduce the disease (Chang, Ward, Gebhart and Kurtz, 1983).

In sheep a similar proliferative disease has been described. Wensvoort (1962) cited for the first time a disease in the Texel breed characterized by poor condition, a reduced growth rate of lambs and a clinical picture of "stretcher" because the animals extended the fore and hind limbs. At this stage, only a macroscopic anatomo-pathological description was described with a thickened terminal ileum and a hypertrophic corrugated mucosa similar in aspect to the lesions found in Johne's disease. Later, Hoorens, Oyaert, Meyvisch, Vandenberghe and Derijcke (1977) described the same disease in 4-month-old Texel lambs but this time more detailed pathological studies were undertaken. On autopsy a thickened jejunum and ileum were found, the appearance of the mucosa was described as cobblestone-like in parts and flattened, thin and rough in others. Histologically, the villi were short or had totally disappeared, the surface of the epithelium was normal in some areas but in others it was desquamated or absent. There was a chronic inflammatory reaction in the lamina propria and serosa infiltrated with lymphocytes, plasmocytes and histiocytes and, in some areas, there was hyperplasia of the glands of Lieberkühn.

On EM examination bacteria of about  $0.2 - 0.3 \mu \times 1 - 1.5 \mu$  were observed in the apical cytoplasm of hyperplastic epithelial cells. The authors found this disease very similar to the one described by Lawson and Rowland (1974) in pigs and suggested that further bacteriological examination was necessary to isolate the intracytoplasmic campylobacter-like bacteria. Three years later, this group of investigators (Vandenberghe and Hoorens, 1980) following similar bacteriological techniques to those previously used for pigs succeeded in isolating Campylobacter spp. from 3 lambs with clinical and pathological lesions of regional enteritis. According to the authors the strains from 2 lambs resembled C. mucosalis except that they did not reduce nitrites. Nevertheless, these strains grew at  $43^{\circ}\text{C}$ , produced  $\text{H}_2\text{S}$  in Kligler iron agar and did not grow on a medium containing cephalothin (Lauwers, De Boeck and Butzler, 1978) so, considering these characteristics it could be possible to suggest that they may well be catalase negative strains similar to "C. fecalis" or perhaps identical to C. sputorum subsp. bubulus which can be isolated from healthy sheep. A simple and accurate test to differentiate between C. mucosalis and these strains would be growth in a microaerophilic atmosphere without hydrogen because C. mucosalis is unable to grow whilst most other campylobacters are not hydrogen dependant (Lawson, Leaver, Pettigrew and Rowland, 1981).

Unfortunately, no more bacteriological studies have been performed with these strains and no comparative surveys between the intestinal microaerophilic flora<sup>of</sup> healthy and diseased sheep. It is very difficult therefore to know if these campylobacters are or are not associated with the intracellular vibrioid bacteria. The third lamb



examined had a diphtheric inflammation of the gut and C. jejuni was isolated. Another group of investigators (Cross, Smith and Parker, 1973) described seven cases of terminal ileitis in 4-6-month-old lambs characterized by the typical rugose thickened ileum which grossly resembled Johne's disease. The histology was similar to the other descriptions and no EM was undertaken. These authors pointed out that as the condition only causes poor weight gain and no deaths it could be easily confused with clinical parasitism and if necropsy is not carried out the disease might occur undetected and perhaps more frequently than it is believed.

Other similar proliferative diseases have been described in weaned hamsters and called wet-tail, proliferative ileitis, regional enteritis, intestinal adenocarcinoma or atypical ileal hyperplasia. Animals with the disease exhibit an early acute enteritis and proliferative changes in the ileal epithelium. Although the cause of the disease is unknown it may be produced experimentally by oral administration of ground ileal suspension from diseased animals. The pathological lesions are very similar to those described in pigs. EM studies in experimentally infected animals detected two different types of intracytoplasmic bacterial cells: one called large organisms (LO) and the other small organisms (SO) (Frisk and Wagner, 1977). The LO are observed alone between the first and ninth day PI and ultrastructurally are similar to intracytoplasmic Escherichia coli or Shigella flexnerii, rods measuring 0.53 to 1.05  $\mu$  wide and up to 2.2  $\mu$  long and, by immunofluorescent antibody techniques it has been identified as E. coli. This microorganism was also isolated from natural cases of the disease and classified as E. coli serotype 0138

closely related to Shigella boydii types 11 and 12 (Wagner, Owens and Troutt, 1973). Later on, between 6 and 14 days PI both intracytoplasmic LO and SO are contained together within the affected enterocytes. The SO are slightly curved rods of 0.23 to 0.48  $\mu$  wide and up to 2.2  $\mu$  long with ultrastructural characteristic similar to C. mucosalis. The intracytoplasmic LO have been seen dividing by binary fission (Wagner, Owens and Troutt, 1973) and EM photographs of enterocytes in anaphase have shown the SO located at both poles of the mitotic cells (Frisk and Wagner, 1977). The campylobacter-like SO have not been isolated from these experimentally infected hamsters. In other experiments with this disease intracytoplasmic organisms were detected as early as day 5th PI and the description of slightly curved bacteria is very similar to that of previously described SO or C. mucosalis in pigs. Hyperplasia developed later at day 10th PI. Hyperplasia is the primary lesion and a secondary inflammatory response began by day 20th PI. The pathological findings resembled very much adenomatosis described in pigs and other animals and were characterized by progressive replacement of mature villus columnar cells by undifferentiated mitotically active crypt cells. The development of lesions was correlated with accumulation of a specific antigen, detected by means of an immunofluorescent serum obtained from hamsters with advanced lesions, in the apical cytoplasm of enterocytes and also in MLN and liver. In these experiments attempts to isolate the bacteria<sup>failed</sup> and the antigen remained unknown (Jacoby, 1978; Johnson and Jacoby, 1978). Some years later, C. fetus subsp. jejuni (jejuni/coli GC) was independently isolated by two teams of investigators from different groups of hamsters with

proliferative ileitis and they considered that this bacterium might be associated with the disease because faecal cultures from unaffected hamsters were negative. Both strains were used in separate experiments to orally inoculate hamsters and the jejuni/coli GC consistently failed to reproduce the disease and the animals remained clinically normal. Nevertheless, it was possible to re-isolate the inoculated bacteria from faeces in high numbers or, from the normal ileum of the animals (Lentsch, McLaughlin, Wagner and Day, 1982; La Regina and Lonigro, 1982).

Proliferative colitis has been detected in European ferrets (Mustela putoris). Clinically, it is characterized by mucohaemorrhagic faeces, prolapsed rectum, anorexia, body weight loss and dehydration. In EM photographs clusters of intracellular comma shaped bacteria located in the supranuclear cytoplasm are seen. The pathological changes resemble the proliferative diseases of pigs, sheep and hamsters but in this case the lesion is restricted to the colon whereas the ileum and small intestine are not affected. C. fetus subsp. jejuni (jejuni/coli GC) was isolated from the faeces of diseased ferrets and no attempt was made to isolate other campylobacters (Fox, Murphy, Ackerman, Prostak, Gallagher and Rambow, 1982). Oral inoculation of ferrets with the isolated strain in high doses per animal ( $2.5 \times 10^8$  CFU) produced transient diarrhoea containing blood and mucus between the 4th and 7th day PI. The inoculated jejuni/coli GC strain could be recovered at levels of  $7.3 \times 10^7$  per gram in faeces but the animals recovered and no histological abnormalities were detected (Fox, 1982; Fox, Newcomer and Ackerman, 1982).

Intracytoplasmic campylobacter-like bacteria within enterocytes

with similar pathology to that of the other diseases previously described, have been recorded in adenocarcinomas of wistar rats (Vandenberghe and Marsboom, 1982), duodenal hyperplasia of a guinea pig (Elwell, Chapman and Frenkel, 1981) and intestinal adenomatosis of the caecum and adjacent colon of a blue fox (Landsverk, 1981). In all cases no campylobacters were isolated.

Intraepithelial vibrios have also been associated with acute typhlitis in rabbits but the brief description of this disease differs from the proliferative enteropathies and degenerative changes are prominent (Moon, Cutlip, Amtower and Matthews, 1974).

One characteristic is common to all these diseases: the presence of intracellular vibrioid bacteria within enterocytes of specific affected areas of the intestines with proliferative changes, these organisms are absent from normal non-affected areas of the same animal and they have never been found within the cells of healthy controls. Whether the campylobacters observed are harmless saprophytes which invade the damaged epithelium but contribute nothing to the pathogenesis of the disease or whether they participate as aetiological agents in combination with other unknown causes, is something that remains to be discovered.

## CHAPTER 2

### GENERAL MATERIALS AND METHODS

#### Plan of work

The preliminary work of this project was to select and develop satisfactory media which would allow the isolation of a wide range of different species of Campylobacter from the faeces of ruminants. Of the media available at that time Skirrow's medium (Butzler and Skirrow, 1979) and Preston's (Bolton and Robertson, 1982) were selected. Initially, the growth of laboratory strains was tested in both media and then, later, the isolation from diagnostic or slaughter house samples of ruminant faeces. As a consequence of this work, a modified Preston medium (PN) was developed and thereafter it was used throughout the work of this thesis for isolation purposes from calves with neonatal diarrhoea and from apparently healthy herds.

A combination of biotyping schemes recently developed (Skirrow and Benjamin, 1980b, Karmali, De Grandis and Fleming, 1980; Karmali, Allen and Fleming, 1981) was used to classify the campylobacter strains isolated during the first year of the research. This scheme was adequate for the recognition and classification of the jejuni/coli GC but it did not satisfactorily sub-divide the other campylobacters. For that reason, during the second year of work a simplified biotyping scheme was used to classify jejuni/coli GC and the other were generically named "fetus group campylobacters" (fetus GC) and were stored at  $-80^{\circ}\text{C}$ . Different procedures during this second year of research considerably increased the isolation of

campylobacters of the fetus GC and it was discovered that different species of campylobacters could be frequently isolated from one animal. Later, further work with a selected group of campylobacters representative of the fetus GC allowed the development of a simplified scheme to classify the stored fetus GC strains. Some of the biochemical and biological tests used through this thesis have been modified from the original methods and the reasons for those changes will be discussed in the relevant chapters.

Comparison between bovine and ovine campylobacters of the fetus GC and, to a lesser extent between porcine and human strains were carried out to clarify the confused taxonomy of this group.

A survey was made on apparently healthy ovines at the Veterinary Field Station of the University of Edinburgh and more detailed studies were performed of the incidence and prevalence of campylobacters in lambs from birth to 3 months of life. This material also provided a number of campylobacter strains for comparative studies.

Recently, fetus GC and jejuni/coli GC strains have been isolated from diarrhoeic calves and were apparently capable of causing symptoms and intestinal pathological changes in conventional calves (Firehammer and Myers, 1981; Al-Mashat and Taylor, 1980a and b, 1981, 1983). In order to assess the possible significance of campylobacters in calves suffering from neonatal diarrhoea a two year survey of the field disease was undertaken. Faeces from scouring and non-scouring calves within affected herds were cultivated for the isolation of different species of Campylobacter and simultaneously processed at the Animal Disease Research Association (A.D.R.A.),

Moredun Institute, for the diagnosis of rotavirus, coronavirus, ETEC and Cryptosporidium sp. These results were also compared with the campylobacter excretion rate of apparently healthy calves from 3 herds without diarrhoea at the time of sampling. These surveys provided the majority of the campylobacter strains used for taxonomic studies and for experimental infections of gnotobiotic ruminants.

The passive haemagglutination technique of Penner and Hennessy (1980) was used to serotype the jejuni/coli GC strains employed to experimentally infect gnotobiotic ruminants and to assess some of the serotypes present in four selected herds.

Finally, a series of experiments were performed on gnotobiotic calves and lambs - as a model for the calf - which included single infections with three of the most common Campylobacter sp. isolates from calf neonatal diarrhoea: C. jejuni, C. coli and CHI.

## 1) Bacteriological Techniques

### 1.1 Culture media, antibiotics and diluents

Agar for tolerance tests: this was prepared according to Skirrow and Benjamin (1980b) and consisted of Blood Agar Base No. 2 (Oxoid CM 271) with the addition of 0.8 per cent (w/v) of Bacteriological Agar (Oxoid L 11) and 1 per cent (v/v) of iron-bisulphite-pyruvate supplement (FBP). The medium was dispensed in 90 mm plastic petri dishes (Sterilin), which were used throughout for agar plates unless otherwise stated, at 3 plates for each 50 ml.

Antibiotics for culture media: all antibiotics except Rifampicin, were dissolved in water to give a concentration of each compound 100



times that finally employed in each medium and stored at  $-20^{\circ}\text{C}$  in vials. Each time a medium was required a vial was thawed and the contents added at 1 per cent (v/v).

- Preparation of Rifampicin: 0.1 gram of Rifampicin (Sigma-R3501) was dissolved in 20 ml of methyl alcohol and dispensed in McCartney bottles in 2 ml amounts. The bottles were stored at  $-20^{\circ}\text{C}$ . When the medium was required 8 ml of distilled water was added to the 2 ml of the alcoholic Rifampicin solution and the solution immediately added at 1 per cent (v/v) to the agar.

Biphasic slopes (CBA/TPB): they were prepared by overlaying a 10 ml 7 per cent (v/v) oxalated horse blood/Columbia blood agar (CBA) slope in a McCartney bottle with 5 ml tryptose phosphate broth (TPB).

Blood agar base plus agar (BABA): Blood Agar Base No. 2 (Oxoid CM 271) incorporating 6 per cent (v/v) of oxalated horse blood and 0.2 per cent (w/v) of Bacteriological Agar (Oxoid L 11).

Brucella semisolid agar (BSA): Brucella Broth (Difco 0495) incorporating 0.3 per cent (w/v) of Yeast Extract Powder (Oxoid L21), 0.15 per cent (w/v) Bacteriological Agar (Oxoid L33) and 1 per cent (v/v) of FBP supplement. The medium was dispensed in 10 ml amounts in 1/2 oz bijoux bottles.

Campylobacter storage medium: Tryptose Phosphate Broth (Oxoid CM283) was prepared according the manufacturers' instructions and after autoclaving, 5 per cent (v/v) sterile horse serum and approximately 17 per cent (v/v) tyndallized glycerol was added. The medium was dispensed in approximately 0.5 ml amounts in screw-lid bottles or tubes of 10 mm of diameter and stored at  $-20^{\circ}\text{C}$  until required.

30  $\mu\text{g}$  Cephalothin discs (C): one hundred discs of 6.25 mm diameter (Whatman No. 1 filter paper) were sterilized by autoclaving in a

1/4 oz bijoux bottle. Three mg of cephalothin (Glaxo) was dissolved in 1 ml of sterile distilled water and added to the 100 discs. Discs were stored wet at +5°C.

Columbia blood agar (CBA): Columbia Blood Agar Base (Oxoid CM 331) with 5 per cent (v/v) oxalated horse blood.

Columbia blood agar slopes (CBA-slopes): Columbia Blood Agar Base (Oxoid CM 331) with 7 per cent (v/v) oxalated horse blood. The medium was dispensed in 30 ml amounts in 100 ml medical flat bottles.

Horse oxalated blood (Gibco 102-05)

Horse blood (lysed): oxalated blood was stored frozen and before use heated at 56°C in a water bath for 2 hours with periodic mixing.

Iron-bisulphite-pyruvate (FBP) supplement: this was prepared by dissolving 5 grams each of ferrous sulphate  $\text{Fe} \cdot \text{SO}_4 \cdot 7\text{H}_2\text{O}$  (Analar, Hopkin and Williams LTD), sodium pyruvate  $\text{CH}_3 \cdot \text{CO} \cdot \text{COONa}$  (Light and Co.) and sodium metabisulphite  $\text{Na}_2\text{S}_2\text{O}_5$  (BDH) in 100 ml of distilled water. The supplement was kept at +5°C in a bottle protected from the light and used within 3 months of preparation. One ml of this solution was added to 100 ml of the appropriate medium to give a final concentration of 0.05 per cent (w/v) of each chemical (Skirrow and Benjamin, 1980b).

Iron medium ( $\text{H}_2\text{S}$ -FBP): this was made as described by Skirrow and Benjamin (1982). The medium was composed of Nutrient Broth No. 2 (Oxoid CM 67) containing 0.12 (w/v) Agar Technical No. 3 (Oxoid L 13) and 1 per cent (v/v) of FBP supplement. The medium was dispensed in 1/4 oz bijoux bottles filling to the neck.

MacConkey agar (without salt) (Oxoid CM 7b)

Methyl-green DNase agar (MG-DNase): DNase agar (Oxoid CM 321) was

prepared to the manufacturers' instructions but supplemented with a 0.5 per cent (w/v) aqueous solution of methyl-green (MG) that had been repeatedly extracted with chloroform (Smith, Hancock and Rhoden, 1969). One ml of MG was added to 100 ml of medium which was then placed in a water bath at 56°C for 30 minutes before autoclaving to allow evaporation of residual chloroform.

Minca-IsoVitalex agar (Minca-Is): this was prepared according to the method of Guinee, Veldkamp and Jansen (1977) by Dr. D. Sherwood, A.D.R.A.

Modified Brewer thioglycollate medium (TM): Nutrient Broth (Oxoid CM 1) incorporating 0.11 per cent (w/v) sodium thioglycollate (BDH) and 0.05 per cent (w/v) "Ionagar" No. 2 (Oxoid L12). The medium was dispensed in 20 ml amounts in McCartney bottles.

30 µg nalidixic acid discs (Nal): sensitivity discs (Oxoid NA-30).

Nitrite medium: Brucella Broth (Difco 0.495) incorporating 0.3 per cent (w/v) of Yeast Extract Powder (Oxoid L21) and 0.001 per cent (w/v) of  $\text{NaNO}_2$ . The medium was dispensed in 5 ml amounts in 1/2 oz bijoux bottles and autoclaved at 115°C for 20 minutes.

Novobiocin-brilliant green-trimethoprim agar (NBGT): prepared as the RNBGT medium without the addition of rifampicin.

Nutrient agar slopes (NAS): Nutrient Agar (Oxoid CM3) dispensed in 5 ml amounts in 1/2 oz bijoux bottles.

Nutrient broth (NB): Nutrient Broth No. 2 (Oxoid CM 67) dispensed either in 5 ml or 0.5 ml amounts in 1/4 oz bijoux bottles.

Nutrient broth with iron-bisulphite-pyruvate supplement (NB-FBP): Nutrient Broth No. 2 (Oxoid CM67) plus 1 ml per cent (v/v) of FBP supplement dispensed in 0.5 ml amounts in 1/4 oz bijoux bottles.

Phosphate buffered saline (PBS): PBS (pH 7.2, 0.1M) and PBS (pH 7.2,

0.01M) were prepared from stock solutions according to Cruickshank, Duguid, Marmion and Swain (1975).

Preston-nystatin agar (PN): a modification of the medium described by Bolton and Robertson (1982) with the following formula:

Nutrient Broth No. 2 (Oxoid Cm 67) incorporating  
 1.2 per cent (w/v) Bacteriological Agar (Oxoid L 11)  
 Polymyxin B Sulphate (Sigma P-1004) ..... 5 IU/ml  
 Trimethoprim (Sigma T-7883) ..... 10 ug/ml  
 Rifampicin (Sigma R-3501) ..... 10 ug/ml  
 Nystatin (Sigma N-3503)..... 100 IU/ml  
 1 per cent (v/v) iron-bisulphite-pyruvate supplement (FBP)  
 6 per cent (v/v) oxalated, lysed horse blood

The nutrient broth and the bacteriological agar were added to distilled water, boiled to dissolve and then autoclaved. The solution was cooled in water bath at 56°C and the other ingredients were added.

Preston-nystatin-Columbia-blood agar (PN-CBA): this was prepared as indicated for PN replacing the nutrient broth and bacteriological agar by Columbia Agar Base (Oxoid CM 331).

Rifampicin-novobiocin-brilliant green-trimethoprim agar (RNBGT):

modified from the original of Lawson and Rowland (1974) (novobiocin-brilliant green agar, NBG), it has the following formula:

Columbia Agar Base (Oxoid CM 331) incorporating  
 1 per cent (w/v) yeast extract (Oxoid L 21)  
 Rifampicin (Sigma R-3503) ..... 5 ug/ml  
 Novobiocin ..... 5 ug/ml  
 Trimethoprim (Sigma T-7883) ..... 5 ug/ml  
 Brilliant Green (GURR) 1 in 80,000

5 per cent (v/v) oxalated, lysed horse blood

The Columbia agar and the yeast powder were added to distilled water, boiled to dissolve and then autoclaved. The solution was cooled in a water bath at 56°C and the other ingredients were added. Batches of medium were tested as described by McCartney (1982).

Ringer's solution (Oxoid BR 52): a quarter-strength Ringer's solution was dispensed in 0.5 ml amounts in 1/4 oz bijoux bottles.

Sabouraud dextrose agar plates: Sabouraud agar (Oxoid CM 41).

Sabouraud liquid medium (Oxoid CM 147): this was dispensed in 5 ml amounts in 1/2 oz bijoux bottles.

Skirrow's agar (S): Blood Agar Base No. 2 (Oxoid CM 271) was boiled to dissolve, autoclaved and cooled at 56°C. Seven per cent (v/v) oxalated horse blood, 1 per cent (v/v) Campylobacter Antibiotic Mix (Gibco Europe 141-0046) and 1 per cent (v/v) of FBP supplement were incorporated.

Skirrow's agar plus agar (SA): prepared as Skirrow's agar (S) with the addition of 0.2 per cent (w/v) of Bacteriological Agar (Oxoid L 11).

Skirrow's nystatin agar (SN): prepared as Skirrow's agar (S) incorporating 100 IU/ml of Nystatin (Sigma N-3503).

Transport medium:

Nutrient Broth (Oxoid CM 1) .....	1.3	g
Proteose Peptone (Oxoid L 46) .....	1.5	g
Sodium thioglycollate .....	0.05	g
Agar (Ionagar No. 2, Oxoid L 12) .....	0.5	g
Distilled water .....	100	ml

The agar was added to a small amount of water, dissolved by boiling

and the rest of the components previously dissolved in water at  $56^{\circ}\text{C}$  added. The medium was dispensed in 1/4 oz bijoux bottles filled to the neck, autoclaved at  $115^{\circ}\text{C}$  (10 lbs/sq.inch) for 20 minutes with the screw-lids loose. After autoclaving the screw-lids were tightened.

2,3,5-triphenyl-tetrazolium chloride strips (TTC): strips of 7-7.5 cm by 0.9-1 cm were cut from Ford's Gold Metal blotting paper, sterilized by autoclaving and then soaked in an aqueous solution of 4 per cent (w/v) of Tetrazolium Salt (BDH). The strips were placed on glass and dried at  $60^{\circ}\text{C}$ . The TTC strips were stored at  $+5^{\circ}\text{C}$  protected from exposure to light.

Triple sugar iron agar (TSI) (Oxoid CM 277): this was dispensed in 15 ml amounts in McCartney bottles.

Tryptose phosphate broth (TPB) (Oxoid CM 283): dispensed in 5 ml amounts in 1/2 oz bijoux bottles.

Yeast extract nutrient broth medium (YNB): it was prepared according to Razi, Park and Skirrow (1981) and supplemented with  $\text{KNO}_3$ , potassium L-aspartate or sodium fumarate as required. After the addition of one of the supplements the broths and the unsupplemented control were adjusted to pH 7.2 with NaOH (N). Media were distributed in 15 ml amounts in McCartney bottles, autoclaved and thereafter cooled to  $42^{\circ}\text{C}$  before inoculation; other batches of media were distributed in 2 ml amounts in 9 x 65 mm tubes plugged with cotton wool, autoclaved and cooled to  $42^{\circ}\text{C}$ .

## 2) Sterilization

In this thesis, "autoclaved" or "sterilized by autoclaving" means

exposure to  $121^{\circ}\text{C}$  (15 lbs/sq.inch) for 15 minutes unless otherwise stated. In general, media, diluents and material were sterilized by this procedure. When other method of sterilization was employed it will be clearly indicated.

### 3) Storage of media

All media dispensed in petri dishes and TSI were stored at  $+5^{\circ}\text{C}$  or  $+7^{\circ}\text{C}$ . Diluents, NAS and CBA slopes and other liquid or semisolid media were stored at room temperature. Iron and NB-FBP media were stored at room temperature protected from light exposure.

Fresh CBA and agar for tolerance tests plates were used within 3 days of preparation. MacConkey, PN, SA, SN and Sabouraud plates, TSI agar and YNB broth were used within 15 days of preparation, unless otherwise stated. All other media were used within two months of preparation.

### 4) Incubation Procedures

#### 4.1 Temperatures of incubation

The temperatures used were  $25^{\circ}\text{C}$ ,  $32^{\circ}\text{C}$ ,  $37^{\circ}\text{C}$  and  $43^{\circ}\text{C}$ . The incubation temperatures of  $32^{\circ}\text{C}$ ,  $37^{\circ}\text{C}$  and  $43^{\circ}\text{C}$  were obtained by means of incubators. The temperature was held between  $42.5^{\circ}\text{C}$  and  $43^{\circ}\text{C}$  and it was not allowed to rise above  $43^{\circ}\text{C}$ . Incubation at  $25^{\circ}\text{C}$  was used only for temperature tolerance tests and was carried out placing jars in a constant  $25^{\circ}\text{C}$  water bath in a cold room ( $+2^{\circ}\text{C}$  to  $+5^{\circ}\text{C}$ ). The level of the water came just below the lid of the jar



and the lid of the water bath was covered with cloth.

#### 4.2 Atmospheres

Aerobic atmosphere ( $O_2$ ): this was used for isolation of aerobes or as a confirmation of strict microaerophilic growth of the isolated campylobacters.

Anaerobic atmosphere (ANA- $O_2$ ): plates and bottles were placed in Mackintosh and Fildes Anaerobic Jars and the jar with fresh catalyst was evacuated to -700 mm of mercury. Hydrogen was allowed to fill the jar, after 30 minutes more hydrogen was added and 10 per cent of the atmosphere removed and replaced by carbon dioxide ( $CO_2$ ). All screw-caps were replaced by cotton plugs.

Strict anaerobic atmosphere (ANA- $O_2$ -plus): jars were prepared as above without secondary evacuation and the addition of carbon dioxide. Additional hydrogen was added to replace the vacuum after 2 hours and overnight incubation at  $37^\circ C$ .

Microaerophilic atmospheres: plates and bottles were placed in anaerobic jars without a catalyst. All screw-lids were replaced by cotton plugs. Three different microaerophilic atmospheres were employed:

- 650  $H_2$ : the jar was evacuated to -650 mm of mercury, allowed to stand for 10 minutes and the negative pressure checked before adding hydrogen to atmospheric pressure. Then, 10 per cent of the atmosphere was removed and replaced by  $CO_2$ .
- 550  $H_2$ : the jar was evacuated to -550 mm of mercury, thereafter 650 procedure was followed.
- 650  $N_2$ : the same method of 650  $H_2$  was followed except for the replacement of the hydrogen by nitrogen.

Combination of temperatures of incubation and atmospheres: different combinations of temperatures and atmospheres were used throughout the work. They will be expressed as the temperature followed by the abbreviation of the atmosphere as for example:  $43^{\circ}\text{C}-550\text{H}_2$ ,  $37^{\circ}\text{C}-650\text{H}_2$ ,  $37^{\circ}\text{C}-\text{ANA}-\text{O}_2$  or  $32^{\circ}\text{C}-\text{O}_2$ .

## 5) Biochemical and Biological Tests

5.1 Catalase test: Overnight NAS cultures incubated at  $43^{\circ}\text{C}-550\text{H}_2$  or NAS cultures incubated at  $37^{\circ}\text{C}-650\text{H}_2$  for 2 days were flooded with 3 per cent (v/v)  $\text{H}_2\text{O}_2$  and examined immediately and then after 5 minutes for the development of bubbles (figure 5.8).

5.2 Temperature growth tests: For each strain to be tested an inoculum from a 2-day-old CBA culture incubated at  $37^{\circ}\text{C}-650\text{H}_2$  was lightly inoculated by means of a wire loop onto a quadrant of a petri dish. The cultures were inoculated onto 3 separate plates and one incubated at each temperature  $43^{\circ}\text{C}-550\text{H}_2$  for 18 hours,  $37^{\circ}\text{C}-650\text{H}_2$  for 48 hours and  $25^{\circ}\text{C}-650\text{H}_2$  for 96 hours.

5.3 Hydrogen dependance of growth: Each strain to be tested was seeded in the same way as for the temperature growth test onto a quadrant of a petri dish and incubated at  $37^{\circ}\text{C}-650\text{N}_2$ .

5.4 Swarming: To demonstrate swarming, freshly made CBA plates were used within three days of preparation, stored at  $+5^{\circ}\text{C}$  or  $+7^{\circ}\text{C}$ . Swarming was evaluated simultaneously with the growth test. The first reading was recorded with the plates incubated overnight at  $43^{\circ}\text{C}-550\text{H}_2$  and the strains which did not grow at  $43^{\circ}\text{C}$  were read at the second day of incubation at  $37^{\circ}\text{C}-650\text{H}_2$ .

### 5.5 Hydrogen sulphide production:

- TSI: the slopes were inoculated from a 2-day-old culture incubated at  $37^{\circ}\text{C}$ -650 $\text{H}_2$  for 2 days. First, the slope was heavily inoculated with a loopful of growth and then the butt was stabbed with a straight wire. Blackening of the butt with or without blackening of the slant was considered as a positive test. Blackening only on the surface of the slant or no blackening was regarded a negative test (figure 5.3).
- Iron medium: growth from half a petri dish was scraped into a lump and removed from the plate with a wire loop. Care was taken to minimise exposure of the campylobacter plate growth to air and inoculation of  $\text{H}_2\text{S}$ -FBP medium was carried out within 10 minutes of opening the jars. The lump was placed at the bottom of an  $\text{H}_2\text{S}$ -FBP bottle, the screw-cap was tightened and the bottle was immediately incubated at  $32^{\circ}\text{C}$ - $\text{O}_2$  overnight. Blackening around the lump of growth was considered as a positive test (figure 5.2).

5.6 Coccal transformation: This was performed according to Karmali, Allen and Fleming (1981). The campylobacters were grown on CBA plates incubated at  $37^{\circ}\text{C}$ -650 $\text{H}_2$  for 48 hours and then the plates were left on the bench in air at room temperature for 6 days. This was used as the routine test.

- Delayed coccal transformation: this was performed in the same way as the coccal transformation but the plates were left on the bench for longer periods. This test was used only for some specific work.

5.7 Hippurate hydrolysis: The rapid test described by Hwang and Ederer (1975) and modified by Skirrow and Benjamin (1980a) from the technique applied to campylobacters by Harvey (1980) was used in this study. A 3.5 per cent (w/v) solution of ninhydrin was prepared in a 1:1 mixture of acetone and butanol, stored in the dark at

+5°C and used within a month of preparation. Campylobacters were grown on CBA plates at 43°C-550H<sub>2</sub> for 18 hours or at 37°C-650H<sub>2</sub> for 48 hours. A loopful of growth was suspended in 2 ml distilled water dispensed in a 1/2 oz bijoux bottle; 0.5 ml of 5 per cent (w/v) recently prepared solution of sodium hippurate (BDH) was added and the mixture was incubated for 2 hours in a 37°C water bath. One ml of the ninhydrin solution was added to the mixture and the bottles were sloped and read first after 10 minutes and then after 2 hours of incubation at room temperature. A deep purple colour as dark as crystal violet was considered as a positive test (figure 5.1, bottle E). Colourless reactions were recorded as negative (bottles A and B). Occasionally doubtful results appeared (bottle C) and the test was repeated. Consistent weak reactions were considered as weakly positive (bottle D).

5.8 Tolerance tests: These were carried out according to Skirrow and Benjamin (1980b). A loopful from an overnight CBA growth at 43°C-550H<sub>2</sub> or from a two day growth at 37°C-650H<sub>2</sub> was suspended in NB-FBP medium. Five equidistant parallel lines were drawn on the base of the agar plate to be used and a further line at right angles down the middle. In this way, 10 strains of campylobacter were tested per plate. A swab was charged with bacterial suspension, touched on the side of the tube to drain excess fluid and then drawn across the designated areas on each of three plates which were inoculated sequentially. Within 15 minutes a TTC strip was placed in one of the plates, aligned with the perpendicular line and five Nal and C discs per plate were placed on the intersections of the lines in the other plates (figure 2.1).

The plates were incubated at 37°C-650H<sub>2</sub> for 42 hours. Zones of

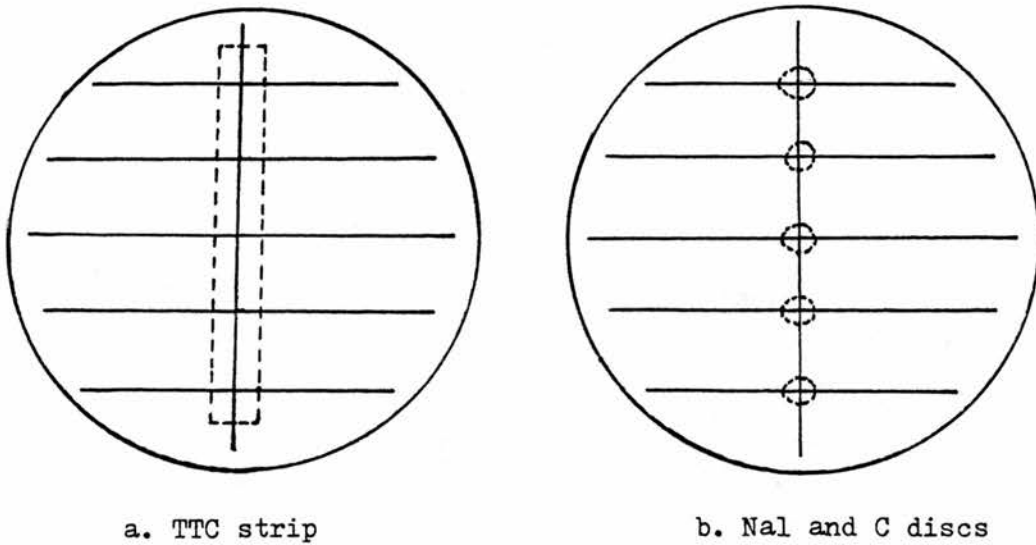


Figure 2.1 Plates for tolerance tests

inhibition to the edge of the strip or disc were measured in mm with a ruler. The appearance of single colonies or two different inhibition areas was considered as a mixture of campylobacters or a contaminated strain and the test was repeated after cloning of the cultures.

#### 5.9 Anaerobic growth in presence of nitrate, aspartate or fumarate:

After autoclaving bottles and tubes were cooled to  $42^{\circ}\text{C}$  in a water bath. A 48 hours CBA campylobacter growth was densely suspended in Ringer's solution. While the agar test media were still melted, supplemented and unsupplemented tubes and bottles were inoculated in duplicate by adding to each 3 drops of the suspension. Bottles were incubated at  $37^{\circ}\text{C}-\text{O}_2$  with lids tightly screwed down whilst tubes were incubated at  $37^{\circ}\text{C}-\text{ANA}-\text{O}_2$ -plus. Cultures were examined after 40 hours, 72 hours and 7 days of incubation. Good growth throughout the supplemented but not in the unsupplemented media was recorded as positive. Growth restricted to the sub-surface layer was recorded as negative.

5.10 Nitrite reduction: A loopful of a two-day CBA growth at  $37^{\circ}\text{C}$ -650H<sub>2</sub> was suspended in NB-FBP medium. Nitrite medium was inoculated with 3 drops of this suspension and incubated at  $37^{\circ}\text{C}$ -650H<sub>2</sub> for 15 days. Nitrite reagents (0.8 per cent (w/v) in 5 N-acetic acid and 0.5 per cent (w/v) alpha-naphthylamine in 5 N-acetic acid) were added to half a volume of the incubated medium as recommended by Cowan and Steel (1974). If a negative result was recorded the remaining half incubated nitrite medium was re-incubated for further 15 days before the final test.

5.11 Other biochemical tests: These were carried out according to methods of Cowan and Steel (1974) to assist in the identification of bacteria other than Campylobacter spp. The oxidase test was performed only on some reference strains of Campylobacter spp. grown on NAS slopes by the method of Kovács (Cowan and Steel, 1974).

## 6) Storage of Bacteria

6.1 Storage of campylobacters: Cultures were grown overnight at  $43^{\circ}\text{C}$ -550H<sub>2</sub> or at  $37^{\circ}\text{C}$ -650H<sub>2</sub> on CBA plates. The plates were heavily inoculated in two separate halves, the growth in each half was scraped into a lump and removed from the plate with a wire loop. The lump of bacteria was placed into the campylobacter storage media without dispersing it. Within 20 minutes of preparation the culture was stored at  $-80^{\circ}\text{C}$ . To recover the organism from the frozen state, the vial was thawed at room temperature and immediately the lump of bacteria was taken and inoculated onto a CBA plate, discarding the remaining medium.

6.2 Storage of other bacteria: Cultures were grown overnight at 37°C in NAS and were kept at +5°C.

7) Transport of campylobacters

Transport medium was heavily inoculated with a loopful of young campylobacter growth from CBA. The bottles were incubated for 1 day at 37°C-650H<sub>2</sub> with screw lids loose. After incubation the lids were immediately tightened, sealed with plastic tape and dispatched.

8) Faecal samples from conventional or gnotobiotic ruminants

Samples from calves and adult ovines were taken from the rectum with separate disposable gloves. In small lambs defecation was provoked with a sterile swab. In all samples, more than 0.5 grams of faeces were obtained. Samples from diagnostic material which were not taken by the author consisted of faeces or swabs. Most of the samples from A.D.R.A. were faeces.

8.1 Direct culture of faeces for isolation of campylobacters:

The procedure was different for each inhibitory medium. When PN was employed, a swab was heavily charged with faeces and approximately 0.5 grams of faeces were extended over 1/5 of the area of the plate. In the case of liquid faeces, around 0.25 ml was introduced onto the area of previously dried PN plates. In order to avoid overgrowth due to contaminants in less inhibitory media - SN or RNBGT - faeces were very lightly inoculated onto the surface of the plates. In all cases the plating out was made by further 5 successive series



of strokes with the loop sterilized between each sequence.

#### 9) Campylobacter strains

Table 2.1 summarizes the campylobacter strains used for laboratory experiments and as controls. Table 2.2 shows the campylobacter strains routinely used as controls in biotyping schemes.

#### 10) Staining techniques

10.1 Capsular staining: It was performed following the method of Chatterjee and Neogy (1972).

10.2 Dilute carbol fuchsin: According to Cruickshank, Duguid, Marmion and Swain (1975).

10.3 Giemsa: This method was used for detection of Cryptosporidium sp. in faeces. Thin smears of faeces or faecal suspensions in PBS were made on glass microscope slides, air dried, fixed in methanol for 2 minutes and then immersed in Giemsa stain (45 ml distilled water, 2.5 ml Giemsa stain, 2 ml methanol and 0.3 ml of 1.5 per cent (w/v)  $\text{NaCO}_3$ ) for 1 hour. The smears were carefully rinsed in tap water, air dried and examined at x 1,000 magnification by light microscopy. Similar technique was used for detecting cells diluting the Giemsa stain approximately 1/10 in tap water. The examination for Cryptosporidium sp. was carried out by Dr. D. Sherwood or Miss I. Campbell, A.D.R.A.

10.4 Gram's method: 0.5 per cent (w/v) of methyl violet in distilled water, Gram's iodine and dilute carbol fuchsin (Cruickshank, Duguid, Marmion and Swain, 1975).

## 11) Virological techniques

Examination for rotavirus or coronavirus in faecal samples of calves with neonatal diarrhoea was carried out by Miss I. Campbell, A.D.R.A.

11.1 Rotavirus-enzyme linked immunosorbent assay (ELISA): The method was that used by Fahey, Snodgrass, Campbell, Dawson and Burrells (1981).

11.2 Coronavirus haemadsorption-elution-haemagglutination assay (HEHA): The procedure of van Balken, de Leeuw, Ellens and Straver (1978) was followed.

## 12) Methods for isolation and identification of K99<sup>+</sup> Escherichia coli from faeces

K99<sup>+</sup> E. coli were identified by Dr. D. Sherwood, A.D.R.A. Recently collected faeces were plated on MacConkey agar. After overnight incubation at 37°C-O<sub>2</sub> 5 representative lactose fermenting colonies were subcultured on Minca-Is agar. After overnight incubation at 37°C-O<sub>2</sub> each of the 5 isolates were suspended in duplicate drops of saline solution on a microscope slide. One drop of undiluted rabbit anti-K12:K99 adsorbed serum (adsorbed with K12 antigen) was mixed thoroughly with one preparation. The other suspension acted as a control for auto-agglutination. Bacteria were allowed to react with the antiserum for 10 seconds before the test result was recorded. Any isolate that agglutinated in adsorbed anti-K12:K99 serum was further tested using sera raised to strains EC6, EC46 and B44 (Sherwood, 1982). Previous work by Sherwood (1982)

had shown almost complete correlation between the presence of K99 antigen and toxic production (STa) in E. coli strains isolated from this part of the country.

### 13) Gnotobiotic calves and lambs

All lambs were hysterectomy-derived, colostrum-deprived as described by Hart, Mackay, McVittie and Mellor (1971). Calves were caesarean-derived, colostrum-deprived obtained in a similar procedure as the one described by Mebus, Underdahl and Twiehaus (1972). The cows were previously sedated with 2 per cent xylazine (Rompun, Bayer), then posterior epidural anesthesia and infiltration of the surgical site was performed by means of 2 per cent lignocaine. All animals were maintained under gnotobiotic conditions in plastic isolators and were fed three times a day with evaporated cows milk reconstituted with sterile distilled water.

The hysterectomies and caesareans were performed at A.D.R.A. by Mr. B. Mitchell and Mr. W. Appleyard. The assembling of the isolators and the feeding and daily care of the animals was done in the Gnotobiotic Unit of A.D.R.A. by Mr. R. McVittie and his staff.

13.1 Gnotobiotic calf or lamb campylobacter inocula: All cultures of C. jejuni and C. coli strains were incubated at  $43^{\circ}\text{C}$ -550H<sub>2</sub> for 20 hours and those of the CHI strain were incubated at  $37^{\circ}\text{C}$ -650H<sub>2</sub> for 42 hours. A loopful of CBA growth of the Campylobacter sp. was suspended in NB-FBP medium. One drop of this suspension was inoculated into each of 5 BSA bottles which were incubated with screw lids replaced by cotton plugs. Growth was evident as a turbidity

line just below the surface of the medium. The 5 BSA cultures were mixed in a 100 ml sterile medical flat and then dispensed in 4 ml amounts into 10 ml sterile glass vials which were immediately heat sealed. The vials required for the inoculum as well as spares were introduced into the gnotobiotic isolator. Bacterial counts were performed on one of the spare vials which had been introduced into the isolator and then withdrawn unused after the inoculation of the animal. These counts were carried out by the Miles and Misra technique (1938) on CBA or BABA plates calculating the titre from that dilution which yielded between 1 and less than 30 colonies.

13.2 Gnotobiotic calf or lamb campylobacter inoculation: One or two-day-old lambs were orally inoculated with 1 ml and 1-day-old calves with 10 ml of the Campylobacter sp. BSA culture.

13.3 Necropsy material from gnotobiotic calves or lambs:

- Blood: as soon as the animal was taken from the gnotobiotic isolator two blood samples were conventionally collected from the jugular vein by means of sterile vacuum tubes. Samples were collected in heparin and without anticoagulant for culture and procurement of serum respectively. The sample for bacteriology was immediately placed in a thermal flask with ice and was cultivated subsequent to the necropsy. Care was taken to disinfect the skin carefully with a diluted alcoholic solution of Lugol's iodine in order to prevent contamination of the blood with campylobacters. Serum was obtained from the other sample by centrifugation and this kept at  $-20^{\circ}\text{C}$  for serological studies.
- Gastrointestinal sites and organs: after the sampling of blood terminal anaesthesia was induced by intravenous injection of

sodium pentobarbitone (Sagatal, May and Baker Ltd., Dagenham, England).

The anaesthesia and sampling of necropsy material was made with Dr. D. Snodgrass, A.D.R.A. Care was taken to ensure that the blood to the gut was maintained during the removal of samples to prevent post-mortem artefact as described by Pearson and Logan (1978).

Samples were taken from the following 9 sites which will be identified through the thesis with the designated abbreviations:

Ab : pyloric area of the abomasum

Site 1: duodenum

Site 2: jejunum

Site 3: mid gut

Site 4: lower ileum without Peyer's patches

Site 5: lower ileum with Peyer's patches

Co : colon

Ca : caecum

Re : rectum

When the sampling of intestinal sites was finished one or two MLN and portions of liver, spleen and lung were aseptically taken. The organs were only used for bacteriological studies whereas with the gastrointestinal sites both bacteriological and pathological studies were carried out.

- Samples for bacteriological studies: the intestinal sites, abomasum and caecum were doubly clamped at one or both sides in order to retain the contents and avoid contamination of organs or other intestinal sites. Then, an 8-12 cm piece was cut and placed with the forceps on a sterile tray. Immediately, both extremes were ligated with sterile string. The tied intestinal sites and the

organs were aseptically introduced in 10 x 15 cm pre-sterilized polythene bags (code BA6040, Colworth, Suffolk, England) and kept in a thermal flask with ice. Gastrointestinal sites were processed as soon as possible after sampling and organs were frozen at  $-80^{\circ}\text{C}$  and processed later.

- Samples for histological studies: a piece of bowel 2-3 cm long was cut open, laid mucosal side up on a glass petri dish and gently covered with 10 per cent buffered formalin.
- Samples for scanning electron microscopy (SEM): a piece of bowel 2-3 cm long was immediately frozen in liquid nitrogen.
- Samples for transmission electron microscopy (TEM): tissue was placed in chilled 0.1M cacodylate buffer at  $+4^{\circ}\text{C}$ , then cut into blocks of approximately  $2\text{ mm}^3$  and fixed in 2.5 per cent glutaraldehyde in 0.1M cacodylate buffer, pH 7.3.

13.4 Bacterial counts of gastrointestinal sites and faeces of gnotobiotic calves and lambs: The ligated bowel was carefully washed with PBS 0.01M, pH 7.2. The sample was placed on a sterile tray, the ligatures were removed and then the bowel was cut open. In the case of the small intestine, which normally contained no contents, the unwashed mucosa and surface mucus was removed with a sterile scalpel into a previously weighed bottle. In samples from the abomasum and large intestine, after opening, the majority of the chyme was removed with a sterile scalpel, then a sample of the mucosa obtained with mucus and residual contents in a similar manner to the small bowel. In each case 0.5 to 1.5 gram of paramucosal material was obtained which was then diluted with enough sterile PBS 0.1M, pH 7.2 to obtain a 1/10 dilution of the material. The diluted paramucosa

was homogenized in an MSE homogenizer for 30 to 40 seconds at maximum speed. Further ten-fold dilutions were made up to  $10^{-9}$  in PBS 0.1M, pH 7.2. The surface viable count method of Miles and Misra (1938) was used. The titre was calculated from that dilution which yielded 1 to less than 40 colonies in the case of Campylobacter spp. and Streptococcus spp. and 1 to less than 20 in the case of Bacillus spp., Escherichia coli or Clostridium spp. The colony forming unit per gram was calculated from the arithmetic mean of the counts obtained at each site which varied between two and six replicates. The number of bacteria was calculated and expressed as  $\log_{10}$ . Different media and incubation procedures were employed in different experiments and will be specified in the description of each experiment.

13.5 Histology: Tissue fixed in 10 per cent buffered formalin was embedded in paraffin wax and 5  $\mu$ m thick sections stained with Mayer's haematoxylin and eosin. In some selected sections Young's (Young, 1969) or Kerr's (Kerr, 1938) modification of the Warthin-Starry technique was done to detect campylobacters in tissues.

13.6 Scanning electron microscopy (SEM): The frozen tissue was cleaved with a chilled acetone-cleaned razor blade and placed in 2.5 per cent glutaraldehyde in 0.1M cacodylate buffer (pH 7.3) at  $+4^{\circ}\text{C}$ . Samples were postfixed in 1 per cent osmium tetroxide dissolved in the same buffer, dehydrated through graded acetone and dried in a Polaron E3000 critical point drying apparatus using carbon dioxide as the drying liquid. As soon as possible after drying, the blocks were coated with gold in a Polaron sputtering system and examined on a Cambridge 5150 scanning electron microscope.



The method used for fixation was modified from Pope, Cole, Guentzel and Berry (1979).

13.7 Transmission electron microscopy (TEM): The blocks of tissue were fixed as indicated in the sampling of necropsy material for 24 hours at +4°C and then rinsed in buffer and postfixed in 1 per cent osmium tetroxide in 0.1M cacodylate buffer, pH 7.3, for 1 hour. Afterwards, the blocks were rinsed in buffer, dehydrated through graded acetone and embedded in araldite (Polaron Ltd.). Ultra thin sections were cut at 600-1200 Å using glass knives on a Cambridge-Huxley Ultramicrotome Mark II. The sections were mounted on copper grids and stained as follows:

Saturated uranyl acetate in 50% alcohol .....	15 minutes
10 % ethanol .....	20 seconds
Distilled water .....	20 seconds
Lead citrate, Reynolds (1963) .....	5 minutes
Distilled water	

The preparation of the material for SEM and TEM was done by Mr. N. MacIntyre and Mr. C. Nicolson, University of Edinburgh

#### 14) Serological techniques

14.1 CHI antisera NCTC11562 (124/73-A4): Hyperimmune antisera prepared against whole cells of CHI NCTC11562 (previously named C. coli type I 124/73-A4 by Lawson, Rowland and Wooding, 1976) was obtained from Dr. G.H.K. Lawson, University of Edinburgh. These "OH" sera had been prepared by the inoculation of rabbits with the surface growth from 48 hours blood agar plate cultures suspended in

normal saline. Four intravenous inoculations were made at 3-5 days intervals and the rabbits were bled 7 days after the last injection. The serum used in slide agglutination tests was preserved with 30 per cent (v/v) glycerine (Lawson, Rowland and Roberts, 1976).

#### 14.2 Slide agglutination tests for detection of CHI type I strains:

Campylobacter strains to be tested were grown for 2 days at 37°C-65OH<sub>2</sub> on CBA plates. Bacterial surface growth was emulsified in duplicate drops of 0.85 per cent sterile saline on a clean glass slide. Using a platinum loop a drop of glycerinated antiserum was added to one of the drops of the emulsion and the other was used as a control for autoagglutination. The slide was rocked gently and carefully examined by means of hand lens against a dark background. If no agglutination occurred within two minutes a negative result was recorded.

14.3 Plate agglutination tests: This technique was used either for titration of sera of gnotobiotic animals or to determine the titer of hyperimmune antisera CHI NCTC11562 against different antigen isolates and the homologous strain.

- Preparation of OH antigens: the technique was modified from Lawson, Rowland and Roberts (1975). The organisms were grown for 48 hours on CBA slopes and the growth washed off with 0.3 per cent formol PBS (pH 7.2, 0.01M). The antigen suspension was washed three times by centrifugation at 4,000 rpm for 20 minutes, discarding the supernatant after each wash. The OH antigens were stored as concentrated antigens in 0.3 per cent formol PBS and used within one month of preparation. Immediately before use the antigen preparations were adjusted to a standard density (approximately equal to that of Brown's opacity

tube No. 2) by means of a nephelometer, diluting the antigen in 0.3 per cent formol PBS (pH 7.2, 0.01M). The C. jejuni and C. coli antigens were diluted in 0.5 per cent formol PBS (pH 7.2, 0.01M) as recommended by Butzler (1978) to reduce autoagglutination.

- Agglutination tests: doubling dilutions of hyperimmune CHI NCTC 11562 or gnotobiotic animal sera were made in PBS (pH 7.2, 0.01M) in the wells of World Health Organization plates (WHO plates). Equal volumes (0.5 ml) of the standard diluted OH antigen were added to the serum dilutions and the WHO plates were incubated at 37°C overnight. Antigen controls in PBS were always included. In the case of C. jejuni and C. coli antigens dilutions of sera and control antigens were made in 0.5 per cent formol PBS. The result was recorded as positive agglutination when a cotton-like flocculum appeared on the bottom of the well for agglutination tests with hyperimmune CHI NCTC11562 serum. Positive agglutination for gnotobiotic animal sera titrations were interpreted in a similar way but, as in many cases the flocculum was weak the plates were agitated to disperse the antigen. If agglutination had occurred the bacteria did not re-suspend homogeneously in the liquid and agglutination could be clearly observed by means of a hand lens. If the bacteria were homogeneously suspended with no presence of macroscopic agglutination particles, the test was recorded as negative. In all cases positive tests were recorded with an arbitrary scale between 1 to 4 points for weakly to strongly positive agglutination respectively.

14.4 Absorption of CHI antisera: A complete growth of three heavily inoculated CBA plates, incubated at 37°C-650H<sub>2</sub> for 2 days,

was removed and suspended in a 1/10 dilution of hyperimmune CHI NCTC11562 antiserum. The vial containing the suspension was placed in a rotary mixer for 2 hours at room temperature and the serum was recovered by centrifugation at 4,000 rpm for 15 minutes (Lawson, Rowland and Roberts, 1977).

14.5 Passive hemagglutination technique for jejuni/coli GC:

This test was performed according to the method of Penner and Hennessy (1980).

### CHAPTER 3

#### ASSESSMENT OF ISOLATION TECHNIQUES

##### Introduction

The isolation of campylobacters from faeces requires special selective techniques based either on differential filtration or direct plating on agar containing selective antibiotics. The selection of an appropriate technique which enables the isolation of different species of campylobacters from the intestinal flora of ruminants was crucial for the work of this thesis.

The filtration technique depends on the ability of campylobacters to pass through a 0.65  $\mu$ m pore size filter whereas other thicker intestinal bacteria are retained. As has been pointed out, the first successful isolations of campylobacters were obtained by differential filtration of faecal suspensions. This procedure has the disadvantage of being time-consuming for the examination of large numbers of specimens and it has been demonstrated that this technique selects jejuni/coli GC from a mixed campylobacter faecal flora of ruminants. Using differential filtration, Firehammer and Myers (1981) and Smibert (1965b) isolated only jejuni/coli GC from bovine and ovine faeces, respectively. Although in both works the media employed would have allowed the recovery of C. fetus no isolation of these bacteria was made. Furthermore, in a limited trial with stock cultures of jejuni/coli GC and C. fetus subsp. fetus Firehammer and Myers (1981) found that colony counts of C. fetus subsp. fetus were more reduced by filtration than the counts of jejuni/coli GC. For those reasons, filtration techniques were not considered appropriate for this study.

Selective media had already been described for the isolation of campylobacters from human faeces when this work commenced. Skirrow's medium (Butzler and Skirrow, 1979), Butzler's medium (Butzler and Skirrow, 1979) and Blaser's medium (Blaser, Cravens, Powers and Wang, 1978) are the most widely used in the United Kingdom, Europe and the U.S.A., respectively. Butzler's and Blaser's media were unsuitable for the purpose of this study because both contain 15 mg/l of cefazolin or cephalothin respectively which inhibit the growth of C. fetus subsp. fetus (Karmali, De Grandis and Fleming, 1980). Another new selective medium, Preston, has been recently developed for the isolation of animal and environmental specimens which have a higher degree of contaminant bacteria than human faeces (Bolton and Robertson, 1982). Skirrow's and Preston media were therefore selected for comparative studies with laboratory strains and direct culture of ruminant faeces.

The NBG medium was developed by Lawson and Rowland (1974) for the isolation of C. mucosalis from the intestinal mucosa of pigs suffering from adenomatosis. Later, this medium was improved by the addition of trimethoprim (NBGT, McCartney, 1982) and subsequently, Lawson and McCartney (Personal Communication, 1983) added rifampicin and named it RNBGT. At the beginning of this work the NBGT medium was used for comparative studies with laboratory strains. As a result this medium was considered unsatisfactory for the isolation of jejuni/coli GC and it was not included in a comparative survey with ruminant faeces. Further work described in this thesis reveals the isolation of "C. fecalis" strains from ovine faeces. Direct plating of "C. fecalis" reference and isolated strains grew very

well on RNBGT but they grew very poorly or not at all on Skirrow or Preston - despite their initial isolation on these media. For that reason, the RNBGT medium was included in a limited survey of bovine faeces to assess the presence of "C. fecalis" in this species.

In addition to the selection of a suitable medium, the most appropriate incubation conditions should be chosen in order to isolate a wide range of intestinal species of ruminant campylobacters. An incubation temperature of 37°C has been described as satisfactory for the growth of most species of campylobacters, although to isolate the jejuni/coli GC from human faeces incubation at 42°C or 43°C was recommended in order to increase the selectivity of the media (Butzler and Skirrow, 1979). Both jejuni/coli GC and C. fetus subsp. fetus have been described as causes of enteritis of cattle (Al-Mashat and Taylor, 1980b and 1983b) so the isolation methods should allow the isolation of at least these two campylobacters. It was obvious that 37°C should be selected as temperature of incubation if C. fetus was not excluded, but it was unknown whether this temperature of incubation would reduce the recovery of jejuni/coli GC in comparison with 43°C incubation. For that reason, comparative counts and isolation of faecal campylobacters at 37°C and 43°C were included in the preliminar studies.

The campylobacters which are the subject of this research are microaerophilic bacteria. They require oxygen to grow yet they are unable to grow under air (21 per cent of oxygen). So, these bacteria must be incubated under reduced oxygen tension, preferably with added carbon dioxide. The system used in this work was to partially evacuate an anaerobic jar, without catalyst and replace the volume



with hydrogen then, 10 per cent of the gas is removed and replaced by carbon dioxide. If the initial vacuum is of 550mm of mercury the oxygen is reduced to approximately 6 per cent. If the initial vacuum is of 650 mm of mercury the oxygen is reduced to approximately 3 per cent. Other bacteriologists use nitrogen instead of hydrogen. There were two decisions to take: whether to use hydrogen or nitrogen or whether to use 6 or 3 per cent of oxygen.

From many previous works it is known that jejuni/coli GC, C. fetus and C. sputorum subsp. bubulus normally grow either with nitrogen or hydrogen rich atmospheres (Butzler and Skirrow, 1979; van Palenstein, Helderma and Rosman, 1976; Lawson, Leaver, Pettigrew and Rowland, 1981). Other bacteria as C. sputorum subsp. mucosalis and some human and porcine oral vibrios grow well only in a hydrogen rich atmosphere and if nitrogen is used they grow poorly or not at all (van Palenstein, Helderma and Rosman, 1976; Lawson, Leaver, Pettigrew and Rowland, 1981). For that reason, hydrogen was chosen as it would support the growth of more species than nitrogen.

Six per cent of oxygen is routinely used for isolation of jejuni/coli GC, C. laridis and C. fetus subsp. fetus in most laboratories. Nevertheless, other campylobacters such as the three C. sputorum subspecies and one particular strain of C. fetus subsp. fetus (Hoffman, Krieg and Smibert, 1979) required less oxygen for optimum growth. It was evident that a 3 per cent oxygen mixture should allow the isolation of oxygen-sensitive strains but it was not known if the use of this reduced oxygen tension would diminish the counts of jejuni/coli GC. Comparative bacterial counts on non-inhibitory and selective agars with both oxygen tensions were made before deciding

which one would be more suitable for faecal isolations.

The strains to be tested were selected. C. jejuni, C. fetus subsp. fetus, C. coli, C. laridis and an atypical C. fetus strain isolated from porcine intestinal contents and called at that time C. coli type I 124/73-A4 (Lawson, Rowland and Wooding, 1975). This strain was later reclassified as CHI type 1, NCTC11562 (Gebhart, Ward, Chang and Kurtz, 1983); the fortunate inclusion of this strain allowed the adaptation of appropriate cultural conditions for the isolation of this group of campylobacters which afterwards were found to be very common among bovine faecal isolates. "C. fecalis" should have been included as it has been isolated from ovine faeces (Firehammer, 1965) and it has been described as possible agent of enteritis in cattle (Al-Mashat and Taylor, 1981). Unfortunately, it was not possible to obtain Firehammer's or Al-Mashat and Taylor's strains at the preliminary stage of this work.

Finally, after the selection of a suitable medium and incubation conditions, the survival of campylobacters in calf faeces was evaluated. Nearly all the faeces collected for the Moredun and diagnostic surveys were sent by post and afterwards stored at +5°C, this work therefore was undertaken to assess the effect of storage on isolation.

Four groups of experiments will be described in the sections of this chapter:

- A) Growth of campylobacter strains on selective media and the effect of incubation temperatures and gaseous environment.
- B) Comparison of bovine and ovine faecal isolation rates from Preston and Skirrow's media.

- C) Comparison of bovine faecal isolation rates from Preston-Nystatin and RNBGT media.
- D) Survival of Campylobacter spp. in calf faeces.

### CHAPTER 3: SECTION A

#### GROWTH OF CAMPYLOBACTER STRAINS ON SELECTIVE MEDIA AND THE EFFECT OF INCUBATION TEMPERATURES AND GASEOUS ENVIRONMENT

The purpose of these preliminary experiments was to select the most suitable incubation conditions and media for the growth of different Campylobacter spp., which were likely to be found in ruminant faeces. Additionally, these experiments allowed the author to acquire experience on practical bacteriological work with campylobacter strains and to develop an adaptation of the Miles and Misra (1938) count technique for these bacteria.

#### Materials and Methods

The media, campylobacter strains and incubation procedures were described in Chapter 2. The P1 medium was Preston medium without nystatin and the P2 medium was prepared without nystatin and with half the concentration of rifampicin (5 ug/ml). P1 and P2 contained 100 ug/ml of actidione as recommended by Bolton and Robertson (1982). Single or duplicate Miles and Misra (1938) counts were performed on ten-fold dilutions in PBS 0.1M, pH 7.2, from a 2-day-old CBA/TPB culture of campylobacter strains incubated at 37°C-650H<sub>2</sub>. Plates were incubated at 37°C-550H<sub>2</sub> or 37°C-650H<sub>2</sub> for 2 days and at 43°C-550H<sub>2</sub> or 43°C-650H<sub>2</sub> for 18 hours. Comparisons between different media, atmospheres and temperatures of incubation were made by the arithmetical mean of the logarithmic values of two plate counts or the logarithmic value of only one plate count. When two counts were performed the standard deviation (S) was calculated .

## Results

### Experiment No. 1:

Colony counts of one CHI and four jejuni/coli GC strains were compared at 43°C with atmospheres containing approximately 6 per cent (550H<sub>2</sub>) or 3 per cent of O<sub>2</sub> (650H<sub>2</sub>) on non-inhibitory CBA medium and selective NBGT or Skirrow's (S) media (Table 3.1).

The NBGT medium was unsuitable for the isolation of jejuni/coli GC strains because none of the three C. jejuni or the C. coli strains tested grew on any dilutions. On the other hand, there was no difference between the counts of jejuni/coli GC either on CBA or S media, either at 43°C-550H<sub>2</sub> or 43°C-650H<sub>2</sub>, so the S medium was considered useful for isolation of this group of bacteria.

The CHI NCTC11562 strain grew either with 3 or 6 per cent of O<sub>2</sub> on CBA. Nevertheless, no growth was detected with 6 per cent of O<sub>2</sub> on NBGT medium whereas growth occurred on the same medium with 3 per cent O<sub>2</sub>. On S medium growth took place at both atmospheres although it was evident that counts incubated with 6 and 3 per cent of O<sub>2</sub> had a 0.52 or 0.2 log reduction respectively in comparison with non-inhibitory medium.

### Experiment No. 2:

Four NCTC reference strains and four different combinations of incubation temperatures and atmospheres were comparatively tested (Table 3.2). Blood agar base (BAB) was used as non-inhibitory medium and its performance was compared with S, P1 and P2 media. During this preliminary work C. jejuni, C. coli or C. laridis on fresh BAB or S plates occasionally produced spreading colonies which prevented quantitation.

As previously described no growth occurred at 43°C with the C. fetus subsp. fetus NCTC5850. This strain grew similarly at 37°C either with 3 or 6 per cent of O<sub>2</sub> on S, P1 or P2.

C. jejuni, C. coli and C. laridis gave similar counts on BAB, S, P1 or P2 at any of the four combinations of atmospheres and temperatures. It was noted that colonies incubated at 43°C were larger than colonies incubated at 37°C but the actual number of colonies was consistently similar at both temperatures.

### Experiment No. 3:

BABA and PN were compared with four campylobacter strains and the four combinations of incubation temperatures and atmospheres (Table 3.3).

The CHI NCTC11562 strain grew either with 3 or 6 per cent O<sub>2</sub> at 37°C or 43°C, on BABA medium. Nevertheless, no growth was detected with 6 per cent of O<sub>2</sub> at 43°C (43°C-550H<sub>2</sub>) on PN and although growth occurred at 37°C with 6 per cent O<sub>2</sub> (37°C-550H<sub>2</sub>) a significant reduction of nearly 1 log (0.98) was detected. On the other hand, no difference was found on the 37°C or 43°C counts with 3 per cent of O<sub>2</sub> atmospheres (650H<sub>2</sub>) showing that this strain becomes increasingly sensitive to oxygen when grown on selective media.

No difference was detected for the C. jejuni biotype 1 or C. coli strains tested at any of the four combinations of incubation temperatures and atmospheres. The strain C. jejuni biotype 2 1097/75 (10-537) was inhibited on PN counts dropping between 0.75 to 0.25 log showing that different strains of jejuni/coli GC could have different sensitivity to selective agents. Here, as in the afore mentioned Experiment No.2 it was noted that colonies incubated at 43°C were

larger than colonies incubated at 37°C although the actual number of colonies was the same at both temperatures.

### Discussion

After experiments 1 and 2 it was concluded that S, P1 or P2 were suitable for isolation of jejuni/coli GC, C. fetus or CHI strains.

Media P1 and P2 did not differ in their performance showing that rifampicin had no inhibitory effect on campylobacter counts at the level of 10 ug/ml. Further experiments with ruminant faeces (vide supra) demonstrated the necessity of replacing the actidione by nystatin because of fungal contamination of faecal cultures incubated for up to 7 days. For that reason, PN was used in comparative studies of experiment 3. S, P1, P2 and PN media contained 1 per cent of FBP-supplement as recommended by Skirrow and Benjamin (1980b) to enhance the aerotolerance of jejuni/coli GC (Hoffman, Krieg and Smibert, 1979).

As the FBP compound seemed not to affect the counts of the C. fetus subsp. fetus or CHI strains tested, it was considered advantageous to add the FBP in order to protect and presumably increase, the isolation rate of jejuni/coli GC. Later, during the second year of work, it was discovered that the FBP compound inhibits the growth of some "C. fecalis" and C. sputorum subsp. bubulus strains (Chapter 5). It was concluded that S or PN were satisfactory media in laboratory experiments and the recovery of campylobacters from ruminant faeces should be compared on these media.

In experiments 2 and 3 similar numbers of C. jejuni, C. coli or



C. laridis colonies were found after incubation at 37°C or 43°C although it was observed that at 43°C colonies grew quicker and were larger than at 37°C. For this reason in this work enumeration was carried out after 18 hours at 43°C to avoid fusion of colonies. Richardson, Koornhof and Bokkenheuser (1982) measured the colony diameter of 25 colonies of 9 jejuni/coli GC strains after incubation for 48 hours and demonstrated that the colonial diameter was significantly increased at 42°C in comparison with 37°C ( $p < 0.02$ ) but Miles and Misra's (1938) enumeration of bacteria was identical.

It was evident from the results of experiment 2 and the previous work of other investigators that typical C. fetus subsp. fetus strains would not grow at 43°C so, for that reason, 37°C incubation temperature was selected for isolation purposes. This temperature would also allow the growth of the jejuni/coli GC without diminishing the chances of isolation of this group.

The increased sensitivity to oxygen tolerance of CHI NCTC11562 strain on the three selective media tested showed that a 3 per cent of O<sub>2</sub> (650H<sub>2</sub>) should be used if this group of campylobacters has to be isolated. Besides, jejuni/coli GC also grew convincingly at this oxygen level and C. jejuni, C. coli and C. laridis gave similar counts on BAB, BABA, S, P1, P2 and PN with any of the four combinations of temperatures and atmospheres.

Extra agar had to be added to CBA, BAB or S medium to avoid interference with the counts by spreading growth of C. jejuni, C. coli or C. laridis colonies. The concentration of 0.2 per cent (w/v) Bacteriological Agar (Oxoid L11) was the minimum required to stop the spreading of the strains.

In addition to the quantitation of laboratory cultures extra agar has to be added to media used for enumerating the bacteria present in clinical material or the tissues of experimental animals.

### CHAPTER 3: SECTION B

#### COMPARISON OF BOVINE AND OVINE FAECAL ISOLATION RATES FROM PRESTON AND SKIRROW'S MEDIA

These two surveys were part of a study on campylobacter faecal flora of diarrhoeic and non-diarrhoeic ruminants and the data showed here is restricted to the comparison of both media.

##### 1) Slaughterhouse survey

##### Materials and Methods

Ovine faeces were collected at random on three different occasions from animals of the pens of Gorgie Slaughterhouse, Edinburgh. Faeces were collected from the rectum as described in Chapter 2, except for one sample which was taken from the floor.

Preston medium (P) was prepared according to Bolton and Robertson (1982) replacing the New Zealand agar by the same percentage of Bacteriological Agar (Oxoid L11). Skirrow's (S) medium was prepared according to Butzler and Skirrow (1979). P and S plates were differently inoculated with faeces as described in Chapter 2. All plates were incubated for 7 days; P plates were incubated at  $43^{\circ}\text{C}$ -650H<sub>2</sub> and S plates at  $37^{\circ}\text{C}$ -650H<sub>2</sub>. Jars were re-gassed every one or two days. Plates were examined at the 3rd, 5th and 7th day after incubation (AI) and growth of campylobacters and/or contaminants were recorded. When growth occurred, 4 representative colonies were subcultured onto a quadrant of a CBA plate which was incubated overnight at  $37^{\circ}\text{C}$ -650H<sub>2</sub>. Campylobacter negative plates were subcultured at the 3rd, 5th and 7th day AI but as soon as one campylobacter strain was isolated the positive selective plate was

discarded and no more sub-cultures for campylobacters were made. Both media were followed up independently and the strains isolated from P or S from the same animal were cloned and biotyped separately. Campylobacters were initially identified by their typical morphology in dilute carbol-fuchsin smears, were then cloned on CBA and thereafter frozen in campylobacter storage medium for biochemical tests at a later time. Details of the biotyping will be referred to later on in Chapter 5 and the identification of the species will be summarized here.

### Results

Campylobacters were isolated from 14 out of 74 (18.9 per cent) ovine faeces. Thirteen isolations were made on P and only 4 on S (Table 3.4).

Seven C. jejuni biotype 1 and one C. jejuni biotype 2 strains were isolated from P plates whereas only three of the C. jejuni biotype 1 were isolated from the S plates. "C. fecalis" was isolated from 5 ovines by means of P medium whereas none of these animals was positive on S plates. Nevertheless, "C. fecalis" was isolated by means of S medium from one campylobacter negative sheep on P plates (Table 3.5).

No difference was demonstrated between the media on the appearance of campylobacter colonies. Nine C. jejuni strains were isolated at the 3rd day AI and two were detected on P at the 5th day AI. The five "C. fecalis" from P medium were isolated as follows: two at the 3rd day AI, one at the 5th day AI and two at the 7th day AI. The other "C. fecalis" was isolated from an S plate at the 5th day AI.

The contamination rate on the two media was different. Forty

four P plates had no growth at all of contaminant bacteria or only a few colonies restricted to the area of direct faecal inoculum, 25 P plates had moderate growth of contaminants restricted to the second stroke and only 5 plates were heavily contaminated with fungi. On the other hand, all 74 S plates had moderate to abundant growth of contaminant bacteria up to the 3rd or 4th series of strokes and the plates inoculated with faeces which had grown fungi on P medium also were contaminated by these organisms.

### Discussion

It was clear that the isolation rate for P plates was higher than the isolation rate for S plates because of the lack of bacterial contamination on the former. The P plates were incubated at 43°C whereas the S plates were incubated at 37°C, it was therefore possible that incubation temperature might have had an effect over the bacterial contamination rate.

Five samples were heavily contaminated by fungi which interfered with isolation on both P and S plates. To overcome this problem the actidione of P medium was changed to nystatin and also nystatin was added to S medium.

This survey was carried out simultaneously with the experiments 1 and 2 already described, so at this stage it was not known if the 37°C incubation temperature would or would not diminish the isolation of jejuni/coli GC. In these two experiments the bacterial counts of jejuni/coli GC were similar at 37°C and at 43°C therefore it was concluded that another study should be made before taking a final decision on the media which should be routinely used. Consequently, a survey was undertaken with diagnostic material to compare the

modified Preston-nystatin (PN) with the Skirrow-nystatin (SN) both incubated at  $37^{\circ}\text{C}-650\text{H}_2$ .

## 2) Diagnostic material survey

### Materials and Methods

The material examined had been sent for diagnostic purposes to the Bacteriology Laboratory, Veterinary Pathology Department, Veterinary Field Station, University of Edinburgh. In this comparative survey, 2 ovine faecal swabs, 10 ovine faeces, 12 bovine faecal swabs, 6 bovine faeces and 1 bovine intestinal content were examined. Details of the clinical status and age of these animals will be considered in the complete analysis of the diagnostic material survey.

PN and SN media were prepared and distinctly inoculated with faeces as described in Chapter 2. All plates were incubated for 7 days at  $37^{\circ}\text{C}-650\text{H}_2$  and followed up as described in the slaughterhouse survey. In this survey, both media were examined independently but only one strain of campylobacter per animal from one of the media was cloned and kept frozen for biotyping. Details of biotyping will be referred to later on and the identification of species will be summarized here.

### Results

Campylobacters were isolated from 14 out of 31 (45.2 per cent) bovine and ovine faecal samples. Fourteen isolations were made on PN and in only 9 of these animals the same Campylobacter spp. were isolated on SN. All negative animals on PN were also negative on SN. Eleven isolations were made from bovine samples on PN and only

7 on SN. Three isolations were made from ovine samples on PN and only 2 on SN (Table 3.6).

More C. jejuni biotype 1, C. coli or CHI strains were obtained on PN than on SN (Table 3.7).

Slight fungal contamination was detected on two PN and SN plates inoculated with the same samples, but they did not interfere with the isolation of campylobacters. Bacterial contamination, in three cases swarming Bacillus spp., was present on most of the SN plates whereas the corresponding PN plates had little or no growth of these bacteria restricted to the area of faecal inoculation.

No difference between the media was evident on the time of appearance of the campylobacter colonies either at 3rd, 5th or 7th day AI.

C. jejuni and C. coli were detected between the 3rd and 5th day AI whereas CHI were detected between the 5th and 7th day AI.

### Discussion

It was demonstrated that the isolation rate on PN was higher than for SN, regardless of the species of campylobacter isolated. Since both media were incubated at 37°C it was clear that the incubation temperature had not had any effect upon the bacterial contamination rate. It was also found that there was no advantage in using both media together because the use of a less inhibitory media such as SN did not yield campylobacters from animals negative on PN. The main problem of SN medium was contamination due to Bacillus spp. and other bacteria, some of which had spread by the 3rd day AI preventing the isolation of campylobacters. Bolton and Robertson (1982) and Bolton, Coates, Hinchliffe and Robertson (1983) arrived at similar conclusions while comparing Preston and Skirrow's media.



The SN medium lacked selectivity and did not completely suppress the growth of ruminant intestinal flora in order to allow the recovery of all campylobacter strains. Furthermore, the use of a more inhibitory media as<sup>such</sup> PN did not delay the isolation of different species of campylobacters which were recovered at similar days AI in both media, varying with the natural speed of growth of each species.

To avoid contamination of the SN medium, this was inoculated with less faeces than the PN medium. This difference in technique undoubtedly affected the chances of isolation of campylobacters on SN medium when the number of these bacteria was low, increased inoculum size however would merely have led to higher contamination.

The addition of 100 IU/ml of nystatin did not suppress the appearance of fungal contamination completely but when it occurred the fungal growth was slow and localized to the area of faecal inoculum. By cutting off the contaminated area, agar of the plate could always be reincubated without interference with campylobacter isolation.

It was concluded that PN would be used throughout the surveys for this thesis as a single plate per animal/faecal sample incubated at 37°C-650H<sub>2</sub> for 7 days.

### CHAPTER 3: SECTION C

#### COMPARISON OF BOVINE FAECAL ISOLATION RATES FROM PRESTON-NYSTATIN (PN) AND R.N.B.G.T. MEDIA

The NBGT was considered unsuitable for the isolation of jejuni/coli GC according to the results obtained in Experiment No. 1 (Section A of this chapter). For that reason, this medium was not used during the first year surveys. "C. fecalis" was isolated from ovine samples either from the slaughterhouse (Section B of this chapter) or from the Veterinary Field Station (Chapter 4, Section A) flock, whereas this species was never isolated from bovine faeces. Later on, direct plating of reference and isolated "C. fecalis" strains demonstrated that they grow poorly on PN or SN media, although initially isolated on these media. On the other hand, by direct plating, "C. fecalis" grew satisfactorily on the new RNBGT medium. For that reason in part of the surveys carried out to study the campylobacter faecal flora of diarrhoeic and non-diarrhoeic calves, cultures were made on both PN and RNBGT media to examine whether "C. fecalis" could be isolated from bovine samples and at the same time to compare the performance of both media using the same faecal samples. The data described here is restricted to the comparison of the media and this material is only a part of a survey which will be referred to in the next chapter.

#### Materials and Methods

Two surveys were made for this comparison of media performance:

- 1) Control farms survey: fifty-nine samples from non-diarrhoeic calves on 3 farms without any clinical disease at the time of sampling.

These farms are identified as A, B and C.

2) A.D.R.A. survey: only certain samples from the survey were examined in this way. Here, 93 calves from 15 farms with neonatal diarrhoea were used. Most of these calves were scouring at the time of sampling but a few non-diarrhoeic animals within these affected herds were also obtained. These farms are identified by A.D.R.A. record numbers.

Faeces from the control farms were collected from the rectum as described on Chapter 2. A.D.R.A. samples were received from the Institute and consisted of faeces. PN and RNBGT were distinctly inoculated with faeces as indicated on Chapter 2. All plates were incubated at  $37^{\circ}\text{C}-650\text{H}_2$  except for 24 samples in which duplicate RNBGT plates were incubated at  $43^{\circ}\text{C}-650\text{H}_2$ . PN and RNBGT were incubated for 7 days, the jars were re-gassed every one or two days and no more than 8 plates were placed in each jar. Plates were examined at the 3rd, 5th and 7th day AI and growth of campylobacters and contaminants was recorded. Subcultures were made from 4 representative colonies as indicated previously onto a quadrant of a CBA plate and campylobacters were initially identified by carbol-fuchsin smears from these CBA plates incubated overnight at  $37^{\circ}\text{C}-650\text{H}_2$ . The methodology of work for this survey was different from that previously described for the slaughterhouse and diagnostic surveys. Initial subcultures were made on the 3rd day AI from campylobacter-like colonies, all plates were then incubated and any new campylobacter-like colonies subcultured on the 5th and 7th day AI. The isolated campylobacters were not cloned, they were only purified from contaminants and frozen. From the isolated campylobacters, up to a maximum of two uncloned strains per selective plate were chosen, one of the jejuni/coli GC and one

of the fetus GC (fetus GC generical name to designate either C. fetus, CHI 1 or CHI 2) primarily selected on basis of their different growth on the media. The frozen Campylobacter spp., single or mixtures, were classified with a screening test to be detailed on Chapter 5. For the purpose of this comparative survey, final identification of the species and types will be referred to here.

### Results

By means of the two media, campylobacters were isolated from 103 out of 152 calves (67.8 per cent) (Table 3.10A). Frequently, more than one Campylobacter sp. was isolated from the same calf. Details of the different combinations of campylobacters obtained from the same animals from control farms A, B and C are shown in Table 3.8 and those for the 15 farms of A.D.R.A. survey in Table 3.9. Using both media only one Campylobacter sp. was isolated from each of 68 calves (44.7 per cent) but two and three Campylobacter spp. were isolated from 32 (21.1 per cent) and from 3 calves (2 per cent) respectively (Table 3.10A).

The isolation rate per calf on both media is shown in Table 3.10B. One hundred of the 103 campylobacter positive calves were detected by means of PN plates, this medium used alone would have detected 100 out of 152 calves (65.8 per cent). The cultures on RNBGT yielded only 3 extra animals with campylobacters. Using PN plates 75 calves (49.3 per cent) had one Campylobacter sp. isolated and 25 calves (16.5 per cent) had two species out of the 152 calves investigated. On the other hand, in only 50 out of the 152 calves (32.9 per cent)

were campylobacters isolated on RNBGT. Forty-eight calves (31.5 per cent) had one Campylobacter sp. and in only two calves (1.3 per cent) two species were isolated from one animal. This means that not only was the isolation rate lower on RNBGT than on PN but also the detection of more than one species of Campylobacter was found to be rare by means of RNBGT medium alone, whereas it was relatively frequent on PN plates (Figures 3.1 and 3.2).

Overall 173 campylobacter strains were isolated from 103 calves. One hundred and twenty-one strains (69.9 per cent) were isolated on PN plates whereas only 52 (30.1 per cent) were obtained from RNBGT plates. The results of biotyping the campylobacter strains isolated from PN and RNBGT is shown in table 3.11A and it can be observed that considerably fewer strains of each Campylobacter sp. were obtained from RNBGT than PN plates. RNBGT was less suitable than PN medium for isolation of jejuni/coli GC; 62 (51.2 per cent) and 57 (47.1 per cent) out of the 121 strains isolated on PN were respectively classified as jejuni/coli GC and fetus GC whereas the proportions isolated on RNBGT were 14 (26.9 per cent) and 37 (71.2 per cent) out of the 52 strains isolated.

In order to evaluate the comparative performance of both media further the extra Campylobacter spp. detected by means of one of them, which were not simultaneously isolated from the other, were recorded (Table 3.11B). Fifty-one extra jejuni/coli GC (42.1 per cent) and 33 fetus GC (27.3 per cent) out of the 121 campylobacters isolated by means of PN plates were not found in the corresponding RNBGT plates of the same animals. Considerably fewer extra campylobacter isolations were made by means of RNBGT medium. Only 3 jejuni/coli GC (5.8 per cent) and 13 fetus GC (25 per cent) out of the 52

campylobacters which were isolated on RNBGT plates were not detected at the same time on the PN plates of the same calves. The overall benefit of the use of PN was the detection of 85 extra Campylobacter spp. whereas only 16 extra species were isolated from RNBGT.

In both media most of the Campylobacter spp. were isolated on the 3rd day AI. Without considering the 3 untyped strains, 139 Campylobacter spp. (80.3 per cent) were isolated at the 3rd day AI, 28 (16.2 per cent) at the 5th day AI and only 3 (1.7 per cent) at the 7th day AI. Four C. jejuni and 6 C. coli were isolated from both media at the 5th day AI and 1 C. coli at the 7th day AI. From them only 2 C. coli and 1 C. jejuni were detected on the 5th day AI on PN medium. The remaining delayed jejuni/coli GC were isolated from RNBGT: 3 C. jejuni and 4 C. coli were discovered at the 5th day AI and 1 C. coli was found at the 7th day AI. Considering both media, 11 CHI type 1, 6 CHI type 2 and 1 C. fetus were isolated at the 5th day AI and only one CHI type 1 and one CHI type 2 were isolated at the 7th day AI. The distribution of this delayed fetus GC isolates was similar for both media so no difference on the time of appearance of colonies was found for this group.

Bacterial contaminants were detected on all RNBGT plates whereas only a few contaminant colonies were occasionally found on the corresponding PN and always located in the faecal inoculated area of the plate (Figures 3.1 and 3.2).

"C. fecalis" was not isolated from any plate, not even from any of the extra 24 RNBGT plates incubated at  $43^{\circ}\text{C}$ -65OH<sub>2</sub> which were also all negative for any Campylobacter spp.

## Discussion

In this comparison plates were inoculated with the same faecal samples, were incubated under the same conditions of temperature and atmosphere and subcultures were made on the same days. The only technical difference was the amount of inoculum which was less on RNBGT than on PN plates. This modification was incorporated after a preliminary trial in which it was found that RNBGT plates were not inhibitory enough for ruminant faecal flora. The higher rate of contamination recorded during this experiment confirms the observation and was undoubtedly one of the reasons for the lower isolation rate on RNBGT medium.

In addition to interference by contaminants the comparatively low number of isolations obtained from RNBGT can be explained by campylobacter inhibition. In this survey, it was evident that RNBGT medium inhibited the growth of jejuni/coli GC more than the fetus GC because fewer jejuni/coli GC were isolated from RNBGT than from PN and more delayed isolations were recorded from the former medium. RNBGT contains brilliant green which has been extensively used in the past to isolate fetus GC (Florent, 1959b; Firehammer, 1965) and this dye is probably responsible for the differential inhibition of both groups. El Azhary (1968) cultivated bovine faeces on a brilliant green medium and isolated only fetus GC strains but when the same faeces were simultaneously plated onto a selective medium without brilliant green jejuni/coli GC strains were also recovered.

On the other hand, the high rate of isolation of PN showed that this medium is suitable for isolation of different species. The finding of 25 double Campylobacter spp. on PN plates demonstrated that



this medium originally formulated for jejuni/coli GC also allows the growth of different fetus GC when the primary plates are heavily inoculated with faeces.

The simultaneous use of RNBGT plates allowed the detection of 3 animals considered to be negative for Campylobacter sp. on PN plates. In the 3 calves the isolates were CHI, (Farm A and C, Table 3.8 and Farm E-421, Table 3.9). The other extra 13 Campylobacter spp. isolated on RNBGT plates contributed to increase the number of double and triple species per calf. Thirty-six campylobacter strains isolated on RNBGT proved to be the same species as the corresponding ones on PN plates but interestingly, also 13 different Campylobacter spp. were also classified in the already campylobacter positive calves on PN plates. Furthermore, considering both media together, although the limited number of colonies studied, 32 double and 3 triple Campylobacter spp. isolations were made. This finding clearly shows that<sup>it</sup> is common to find different species of Campylobacter in the same animal and also that the increase in the isolation rate by means of RNBGT medium was partly due to the additional colonies of campylobacter from each animal that were followed up and biotyped.

Finally, no "C. fecalis" was isolated from any RNBGT or PN plates. Although the contamination rate on RNBGT was higher than that on PN plates, it has to be pointed out that, in general, it was insufficient to prevent isolation of reasonably numerous colonies (Figure 3.2). As RNBGT medium is suitable for the isolation directly of "C. fecalis" strains it could be assumed that this bacterium either was not present or only existed in very small numbers in the 152 faeces of calves cultivated.

### CHAPTER 3: SECTION D

#### SURVIVAL OF CAMPYLOBACTER SPP. IN CALF FAECES

Throughout this work it was possible to sample animals and immediately cultivate faeces within few hours only when the material was taken by the author. This was the case for the slaughterhouse and Veterinary Field Station ovine surveys and bovine control farms. All the other bovine and ovine faeces were not cultivated immediately. For instance, some of the diagnostic samples were sent by post and consisted of faeces or swabs. Most of the work with bovine faeces was done on A.D.R.A. samples from calf neonatal diarrhoea outbreaks and for that reason the quality of the material investigated required close attention. These samples consisted of faeces which were stored at A.D.R.A. at  $+5^{\circ}\text{C}$  during a variable period of time before being weekly collected by the author and brought to the bacteriology laboratory, Veterinary Field Station, for their cultivation. Some of these samples had been sent by post and inevitably, for various reasons, some of them suffered more delay than expected before being plated onto the selective medium. The farm sampling data of the 554 A.D.R.A. calf faeces were available and were analysed for any relationship between the age of the faeces at the time of culture and the isolation rate of different Campylobacter spp. In addition, five campylobacter positive A.D.R.A. calf diarrhoeic faeces were stored at  $+5^{\circ}\text{C}$  and periodically cultivated for Campylobacter spp.

#### Materials and Methods

The cultivation of faeces was carried out as described on Chapter 2. Techniques for subculture and examination of plates will be

described in Chapter 4 and biotyping schemes of the campylobacter isolates will be detailed in Chapter 5. All faeces were stored in 1/4 oz bijoux bottles with tightened screw-lids at +5°C.

Statistical analysis of data was done by Chi-square test by the Brandt and Snedecor methods (Snedecor, 1950).

## Results

### 1) A.D.R.A. Survey

The period of time elapsed between the collection of faeces at the farms and their cultivation was calculated for 554 faecal samples from 529 diarrhoeic and 25 non-diarrhoeic calves representing isolates from 75 herds with enteric disease (Table 3.12). The data were grouped into 28 categories, the first one corresponding to less than 1-day-old faeces and the others at 2-day-intervals (Figure 3.3). The distribution of this data is asymmetrical: 68.26 per cent of the samples were collected at  $\bar{x} = 9.2 \pm 7.8$  days and the median value calculated from individual day frequencies is 7 days (Table 3.12). Campylobacters were isolated from nearly all the groups up to 45-46 day-old faeces. The percentage of isolation for each group was calculated and a diminishing recovery was observed as the faeces became older (Table 3.13A). Inspection of the data suggested that the fall in the percentage of campylobacter positive faeces occurred between 5-6 to 7-8 days. Brandt and Snedecor's Chi-square analysis of the groups up to 5-6 days revealed no significant differences ( $\chi^2_{(3)} = 7.629$ ,  $p < 0.1 > 0.05$ ) whereas examination of the groups up to 7-8 days showed a significant difference ( $\chi^2_{(4)} = 17.246$ ,  $p < 0.01 > 0.001$ ). When

the data were pooled (Table 3.13B) a highly significant difference was again detected between the groups ( $\chi^2_{(3)} = 16.680$ ,  $p < 0.001$ ). Comparison between the isolation rates of the 1-6 day-old faeces group and the rest also disclosed a highly significant difference ( $\chi^2 = 15.220$ ,  $p < 0.001$ ).

Two hundred and ninety nine Campylobacter spp. were isolated from the 266 campylobacter positive calf faeces. They were classified as C. jejuni, C. coli, CHI type 1, CHI type 2, C. fetus and untyped. Fourteen untyped campylobacters were detected on CBA subculture plates by means of carbol-fuchsin smears but they were lost due to the presence of contaminants and could not be classified. The isolation of jejuni/coli GC and fetus GC in relation to the age calf faecal groups is shown in figure 3.4. Both groups of campylobacters were isolated from faeces held for long periods after sampling: C. fetus from the 25-26, CHI type 2 from the 29-30, CHI type 1 from the 35-36, C. coli from the 39-40 and the last one, C. jejuni from the 45-46 day-old group. The relative percentages of different Campylobacter spp. distributed into four age calf faecal groups were analysed (Table 3.14). Inspecting the data a reduction in the isolation of fetus GC and an increase of C. jejuni was observed within the first group (less than 1 to 6 days) but this difference was not statistically significant by the Chi-square test neither for C. jejuni ( $\chi^2 = 1.325$ ,  $p < 0.30 > 0.20$ ) nor for fetus GC ( $\chi^2 = 3.389$ ,  $p < 0.1 > 0.05$ ).

## 2) Survival of campylobacters in some A.D.R.A. faeces

Five campylobacter positive diarrhoeic faeces from A.D.R.A. survey were selected at random for the survival test. All were fresh faeces of less than 1-day-old. The consistency of the material was soft

but not liquid. From each faeces single strains of C. jejuni biotype 1 or CHI type 1 or 2 were isolated (Table 3.15). The faeces were subcultured after 9, 16, 31, 38, 45 and 93 days of storage. A C. jejuni biotype 1 and a CHI type 1 positive faeces (No. 1 and 2) had no viable campylobacters after 9 days of storage. Another C. jejuni biotype 1 was recovered from No. 3 faeces up to day 16th of storage. Faeces No. 4 and 5 contained viable C. jejuni biotype 1 and CHI type 2 up to day 93 of storage.

Fungal contamination was found to have increased in all samples cultivated from the 16th day of storage onwards.

### Discussion

The temperature of storage was selected according to information provided by previous reports on survival experiments with a limited number of campylobacter infected human faeces, which demonstrated that at 25°C the faeces become negative within 48 to 96 hours, whereas aliquots of them survived for up to 22 days at +4°C (Tanner and Bullin, 1977; Blaser, Hardesty, Powers and Wang, 1980). Earlier reports indicated that C. fetus organisms survived for 20 days in sheep manure kept at +6°C (Lindenstruth and Ward, 1948) and jejuni/coli GC for 6 days inoculated in previously autoclaved moist calf faeces kept at room temperature (Jones, Orcutt and Little, 1931). The data of the present two studies showed that bovine Campylobacter spp. are able to survive in naturally infected calf faeces for longer periods than the ones previously reported: C. fetus for up to 25-26 days, C. coli for up to 39-40 days and C. jejuni and CHI for more than

93 days.

The time of survival probably depends upon the initial number of bacteria, appropriate environmental conditions, the particular strains of campylobacter considered and the presence of factors lethal to campylobacters. Five human diarrhoeic faeces containing between  $10^7$  to  $10^9$  jejuni/coli GC per gram had no viable organisms after 22 days when kept at  $+4^{\circ}\text{C}$  (Blaser, Hardesty, Powers and Wang, 1980) showing that despite the high numbers these human faeces possibly provided an inappropriate environment for the bacteria. On the other hand, bovine and human C. jejuni strains remained viable for periods of up to 146 days in sterile milk stored at  $+4^{\circ}\text{C}$ , probably due to the strong buffering power of the milk (Redwood, Gill and Lander, 1983). In the bovine faeces studied here the variable number of bacteria present, diverse faecal consistency and different strain resistance could explain why some of the 5 faeces studied became negative by the 2nd subculture while others remained positive for more than 3 months.

The bottles were kept with the screw-lid tightened in order to favour the microaerophilia and also to prevent desiccation. Ullmann and Kischkel (1981) demonstrated that C. fetus and C. jejuni strains were able to survive for 7 days when swabs were dipped into Tarozzi's liver cultures and left at room temperature in glass tubes under atmospheric conditions. According to these results campylobacters show oxygen and desiccation tolerance at least for short periods of time. In this study, the bijoux bottles possibly extended the survival period of the campylobacters.

A statistically significant decline of the faecal isolation rates

was found from the 7th day of storage onwards. This conclusion is very important and has to be taken into account later when comparisons of isolation rates in different surveys are made.

Finally, it was observed in both experiments that the survival period was not correlated with any of the bovine Campylobacter spp. considered. Both jejuni/coli GC and fetus GC strains were able to survive in calf faeces for similar periods and at approximately the same isolation rate.



CHAPTER 4SURVEYS OF CAMPYLOBACTER INFECTIONS IN CATTLE AND SHEEP

The literature review (Chapter 1) has shown the different and sometimes contradictory findings that have been reported in surveys of campylobacter infection. Some investigators isolated campylobacters from non-diarrhoeic ruminants and considered these bacteria as part of the normal bovine and ovine enteric flora (Florent, 1959b; Smibert, 1965a; El Azhary, 1968; Robinson, 1982). Comparison between diarrhoeic and non-diarrhoeic animals have demonstrated apparently opposite results. Prescott and Bruin-Mosch (1981) found that diarrhoeic cattle and sheep less frequently yielded campylobacters than healthy animals whereas Allsup and Hunter (1973) observed that isolations were significantly higher in a "diseased" group of calves than in a "healthy" group. Other researchers have isolated campylobacters from diarrhoeic ruminants but their work included very few or no non-diarrhoeic controls, and after experimental production of disease in conventional calves and lambs they proposed that these microorganisms might be capable of causing enteritis in young ruminants (Al-Mashat and Taylor, 1980a; Firehammer and Myers, 1981).

Comparison shows that the results have probably been influenced by the diverse methodologies used. Furthermore, as has been discussed previously different bacteriological procedures have favoured the isolation of particular species from a mixed campylobacter flora.

In these surveys undertaken by the present author the methodology employed has also influenced the isolation rates. This was noted when the procedure of isolation and biotyping was improved during the second year of work. For that reason the data obtained during these two separate years are not comparable and will be considered separately.

The ovine surveys carried out during the first year of work were a slaughterhouse survey in which ovine faeces were taken at random from non-diarrhoeic sheep, a sequential study of a flock of ewes and lambs and faecal samples submitted for diagnostic purposes. The sheep surveys mainly involved non-diarrhoeic animals and provided material for comparative studies of the campylobacter faecal flora of the two ruminant species.

In the first year the bovine surveys consisted of diagnostic and A.D.R.A. faeces from diarrhoeic herds. During the second year of work the only material studied was of bovine origin, A.D.R.A. survey faeces from diarrhoeic herds and control non-diarrhoeic farms. The purpose of this work was to establish the isolation rates in diarrhoeic and non-diarrhoeic calves in herds with enteric disease and control non-diarrhoeic herds. In this study the presence of other enteropathogens was also established and an examination carried out of the association between the presence of diarrhoea and recovery of particular agents.

Finally the two different methods used to examine bovine faeces were compared with the objective of developing improved bacteriological isolation techniques.

#### Materials and Methods

Faeces were collected and cultivated as indicated in Chapter 2, unless otherwise specified. All PN plates were incubated at 37°C-650H<sub>2</sub> and the jars were regassed every one or two days. Plates were examined at the 3rd, 5th and/or 7th day AI. When growth occurred 4 representative colonies were subcultured onto a quadrant of a CBA plate.

Campylobacters were primarily identified by the typical morphology observed on carbol-fuchsin smears from these CBA plates incubated overnight at  $37^{\circ}\text{C}$ -650H<sub>2</sub>. Campylobacters were provisionally classified as jejuni/coli GC (C. jejuni or C. coli) or fetus GC (C. fetus, CHI or "C. fecalis") according to their colonial and microscopic morphology and later they were biotyped.

Two different bacteriological procedures were used during each of the two year surveys.

#### First year survey procedure

- 1) Up to 12 plates were placed in each anaerobic jar.
- 2) Only one Campylobacter sp. was kept per faeces examined unless different colonial types were evident on selective plates at the 3rd day AI.
- 3) Subcultures thereafter were made on the 5th and 7th day PI from previously campylobacter negative plates. As soon as one campylobacter strain was isolated the selective plate was discarded.
- 4) The isolated campylobacters were cloned and then stored frozen.
- 5) The frozen campylobacters were later classified by a biotyping scheme to be detailed in Chapter 5, Section A.

#### Second year survey procedure

- 1) No more than 8 plates were placed in each anaerobic jar.
- 2) From the campylobacter strains recovered from each faeces up to a maximum of two uncloned strains per plate were chosen, one of the jejuni/coli GC and one of the fetus GC.
- 3) Subcultures at the 3rd, 5th and 7th day AI were made from both previously campylobacter negative and positive plates.
- 4) The isolated campylobacters were only purified from contaminants

and kept frozen without cloning.

- 5) The frozen campylobacters were later classified by a modified biotyping scheme thereafter called the "screening test" which will be detailed in Chapter 5, Section A.

## CHAPTER 4: SECTION A

### OVINE SURVEYS

These surveys were an examination of the campylobacter isolation rates in different flocks and an attempt to demonstrate any relationship with clinical diarrhoea or alteration of faecal consistency. Most of the samples were as it happened obtained from non-diarrhoeic animals. These surveys also provided ovine campylobacters for comparative taxonomic studies of ovine and bovine faecal strains. Additional information was acquired on the Campylobacter spp. excreted by lambs and ewes, the age at which lambs became naturally infected and the possible role of the dam in the transmission of the infection to their off-spring. Parts of two of these surveys were also used to compare bacteriological isolation procedures and have been reported earlier (Chapter 3, Section B).

#### 1) Slaughterhouse survey

This survey was carried out to compare selective media (Chapter 3, Section B), investigate the ovine faecal campylobacter flora and attempt to correlate faecal changes with the presence of different Campylobacter spp.

#### Materials and Methods

Prior to slaughter sheep sent to Gorgie slaughterhouse are retained in holding pens. These sheep are probably derived from many sources and it is possible that some at least have passed through markets in the preceding few days. Sheep were sampled on three occasions in the holding pens at weekly intervals. Methods of sampling rectal faeces are given in Chapter 2, in addition any physical abnormalities were

recorded. Faeces were plated onto P and S selective media as described in Chapter 3, Section B (Table 3.4), following the aforementioned first year procedure. The dentition and breed of the sheep and the appearance and consistency of the faeces were recorded. One rectal faecal sample was collected for each sheep.

Yates' correction for Chi-square was used (Downie and Heath, 1974) to analyse differences in isolation rates.

### Results

Campylobacters were isolated from 14 out of 74 (18.9 per cent) ovine faeces collected in 3 samplings (Table 3.4). Seven faeces (9.5 per cent) contained C. jejuni biotype 1, one (1.4 per cent) C. jejuni biotype 2 and six (8.1 per cent) "C. fecalis" (Table 3.5).

Most of the campylobacter isolations were made from Blackface sheep (Table 4.1). The majority of these animals were aged and in analysis a high proportion of isolations were made among older sheep (Table 4.2). Five Blackfaces excreted C. jejuni biotype 1, one C. jejuni biotype 2 and four "C. fecalis". The remaining Campylobacter spp. were isolated as follows: 2 C. jejuni biotype 1 from a Cheviot (milk teeth) and from a Suffolk (two teeth) and 2 "C. fecalis" from a Cheviot and Border Leicester sheep (both milk teeth).

Faecal changes were detected in 7 sheep (Table 4.3). All the altered faeces had lost their normal pelleted consistency. Five faeces were soft, one was mucoid and one contained red bloody strings. Three C. jejuni and one "C. fecalis" strains were isolated from 4 out of the 7 altered faeces. Statistical Yates' corrected Chi-square comparisons between the animal groups with and without faecal changes were made. An increase was observed of the total campylobacter

isolation rate ( $p < 0.05$ ) within the altered faecal group (Table 4.4A). When C. jejuni and "C. fecalis" were separately considered an increase isolation rate was found ( $p < 0.05$ ) for the C. jejuni group of altered faeces (Table 4.4B) whereas no significant difference was revealed when the "C. fecalis" groups were compared (Table 4.4C).

### Discussion

The relative campylobacter isolation rate varied in the 3 samples 4.8, 20 and 20.6 per cent (Table 3.4). Smibert (1965a) also found variability between different flocks.

In this survey it was found that some apparently healthy sheep excreted one of two species : C. jejuni or "C. fecalis". Jejuni/coli GC have been isolated from apparently healthy non-scouring sheep at similar rates to that found in this survey (10.9 per cent): 16.8 per cent (Smibert, 1965a), 13.6 per cent (Prescott and Bruin-Mosch, 1981) and 13.3 per cent (El Azhary, 1968). Firehammer and Myers (1981) were unable to isolate any campylobacter from diarrhoeic or healthy lambs from 8 ranches.

Mild subclinical faecal changes were detected in a few non-diarrhoeic animals and the analysis of this limited data showed that they might be associated with the presence of C. jejuni. Firehammer and Myers (1981) fed 5 lambs with jejuni/coli GC cultures failing to produce clinical diarrhoea. Instead, the 5 lambs developed mild faecal changes represented by mucoid faeces and intermittent appearance of flecks of blood.

"C. fecalis", indistinguishable from the strains originally described by Firehammer (1965), were isolated from 6 sheep. No correlation between the presence of this species and faecal alterations was found.

Firehammer (1965 and 1979) could not relate "C. fecalis" to any disease. It should be pointed out that both S and P selective media are not fully satisfactory for "C. fecalis" isolation and this bacterium may only have been isolated from a few of the animals excreting the organism. Possibly, the use of a more suitable media for "C. fecalis" would have considerably increased its isolation rate.

Interestingly, more campylobacters were isolated from Blackface sheep. This sheep breed usually lives in isolated highlands and might have less chances to acquire natural campylobacter infection than the other breeds. Non-immune susceptible animals could acquire the infection when they are exposed to overcrowded conditions during the transport or confinement in market pens. Smibert (1965a) isolated jejuni/coli GC from 24 per cent of 14-week to 20-month-old sheep but from only 4.2 per cent of the 2 to 10-year-old sheep. In this work more campylobacters were isolated from old sheep because most of the isolations were made from Blackface animals. No difference between the age of animals and Campylobacter spp. isolation rates was detected.

## 2) Easter Bush flock survey

This survey was initiated on the 8th January 1982 and finished on the 21st May lasting 133 days. Faeces from pregnant Suffolk ewes were cultivated for campylobacters 3 to 21 days before parturition. Faeces from lambs born from the campylobacter excreting ewes were periodically cultivated to assess:

- a) the campylobacter species present in the flock
- b) the age at which lambs acquired infection



- c) the prevalence of lamb infection from 14 to 32 days till 4 months of life
- d) the correlation if any between diarrhoea in lambs and campylobacter excretion.

### Materials and Methods

The pregnant ewes were first sampled when at grass, thereafter animals were housed, separately penned for parturition and for the few following days. Subsequently, ewes and lambs were assigned to four treatment groups unconnected with this work. No attempt was made at any stage to microbiologically isolate animals and the sheep in the four groups were housed in the same building and animals were transferred between groups.

Faeces were cultivated on PN plates and campylobacters were isolated and identified according to the first year survey procedure. Bacteriological methods for detection of K99<sup>+</sup> E. coli (K99<sup>+</sup>EC) were carried out as described in Chapter 2. Ewes and lambs belonged to the Department of Animal Health, Easter Bush, University of Edinburgh. One faecal sample per ewe was collected and lambs were sampled at 13-28 days intervals (Table 4.5) by either rectal swabs or rectal faeces. Minor changes in consistency were not recorded because the ewes were fed on different experimental rations.

Yates' corrected Chi-square was used (Downie and Heath, 1974) in the analysis of data.

### Results

Campylobacters were isolated from 9 out of 29 (31 per cent) ewes. None of the ewes had diarrhoea at the time of sampling. C. jejuni biotype 1 was isolated from 2 and "C. fecalis" from 7 other ewes

(Table 4.5). These animals were identified and their off-spring were periodically cultivated for campylobacters. Sixteen lambs born from these campylobacter positive ewes were followed up.

Lambs 9A and 9B suffered severe diarrhoea and died being 1 and 2 days old respectively. Faeces from these lambs were negative for campylobacters but K99<sup>+</sup>EC were isolated from both animals. The other 14 lambs did not show clinical diarrhoea (Table 4.5).

Campylobacters were isolated from 22 out of 79 (27.8 per cent) lamb faeces. Eleven out of the 14 (78.6 per cent) lambs excreted different jejuni/coli GC and two of them also "C. fecalis". C. jejuni biotype 1 was isolated from 7 faeces, C. jejuni biotype 2 from 2 faeces, C. coli from 10 faeces, "C. fecalis" from 2 faeces and one isolate was not viable on subculturing and could not be typed. Five lambs (1A, 3A, 5B, 6A and 6B) excreted only one Campylobacter spp., 4 lambs (2A, 4A, 8A and 8C) two Campylobacter spp. and 2 lambs (2B and 8B) combinations of three Campylobacter spp. or biotypes. In only 3 out of the 11 lambs excreting Campylobacter spp. the same it bacterium apparently isolated in consecutive samplings (Table 4.5).

In sheep "C. fecalis" was more frequently isolated than jejuni/coli GC whereas in lambs the contrary occurred. In sheep "C. fecalis" was isolated from 7 out of 29 faeces (24.1 per cent) whereas jejuni/coli GC was obtained from only 2 (6.9 per cent) faeces. In lambs jejuni/coli GC was isolated from 19 out of 79 faeces (24 per cent) whereas "C. fecalis" was obtained from only 2 (2.5 per cent) faeces. This difference is highly significant by Yates' corrected Chi-square test ( $\chi^2 = 10.915$ ,  $p < 0.001$ ).

No C. coli infection was detected in any of ewes examined. Five

young lambs of 27-45 days of age acquired C. coli infections (2A, 2B, 8A and 8C) whereas only one of this age group yielded C. jejuni (4A). In four lambs (2A, 2B, 8A and 8C) infection with C. coli preceeded infection with C. jejuni and only in one lamb (8B) infection with C. jejuni preceeded infection with C. coli.

Three of the strains provisionally described here as "C. fecalis" (ewes 7 and 8 and lamb 8B) were catalase negative and could not be differentiated from C. sputorum subsp. bubulus, their taxonomical position will be discussed in Chapter 5.

### Discussion

The possible transmission of campylobacters from dams to their lambs has not been proven as no complete relationship between the Campylobacter spp. isolated has been found. Only 2 out of 11 lambs born from "C. fecalis" carrier ewes acquired the same bacterium. Two out of 3 lambs born from 2 C. jejuni biotype 1 carrier ewes acquired the same species at 91 and 112 days of life respectively and after previous infection with C. coli when 28 days old. The recovery of different Campylobacter spp. from twins or triplets and their corresponding ewes may indicate that direct contact with the dam is not so important as contamination from the environment in the acquisition of infection. Smibert (1965a) also suggested that infection could originate from exogenous infection rather than the dam being the unique source of contamination. Transmission between lambs and their dams was not proven and more studies are needed to assess these relationships.

Lambs' faeces were cultured between 14 to 32 days of life and at this age only 1 out of 16 (6.3 per cent) was infected with C. jejuni biotype 1. It should be pointed out that only swabs could be taken

at this stage so only a small amount of faeces could be plated onto PN plates. Between 28 to 46 days of age the standard amount of faeces was cultivated and 50 per cent of the lambs excreted campylobacters. This finding is important because up to now it has not been known at which age lambs become infected. Smibert (1965a) was not able to isolate any Campylobacter spp. from 10 lambs cultivated weekly from 1 week up to 13 weeks of age although the filtration method used by him detected jejuni/coli GC in sheep older than 3 months. Similarly, Firehammer and Myers (1981) failed to make isolations from diarrhoeic or healthy young lambs. They suggested that lambs could possess a defence mechanism, such as colostral antibody, which prevents colonization at an early age. In the present work it has been demonstrated that lambs can become colonized as early as 28 days of life (Table 4.5). Both previous surveys (Smibert, 1965a; Firehammer and Myers, 1981) used filtration methods which probably were unable to detect low numbers of campylobacters in young lambs whereas in this investigation PN plates were heavily inoculated with faeces.

Flock-to-flock variation in carriage rates has been reported by Prescott and Bruin-Mosch (1981) who found that sheep from two sources gave isolation rates of 23.3 per cent and 2 per cent. In this work the isolation rates varied in the same flock, ranging between 6.3 and 66.7 per cent (Table 4.5). These results are in agreement with Smibert's (1965a) who also found that the infection rate in a flock depends on the number of samples examined from each animal. In this survey, the cumulative infection rate of 14 lambs increased from 7.1 to 78.6 per cent (the faeces of 5 lambs could not be cultivated on the last sampling because they had been sold). It is evident from these

findings that the natural excretion of campylobacters may be for a short period.

Even where the methodology used was limited to the isolation of one Campylobacter spp. per selective plate, different faecal samples from the same lambs yielded different species. Six out of the 11 campylobacter positive lambs (54.5 per cent) shed two or three Campylobacter spp. (Table 4.5). Mixed natural campylobacter colonization of lambs should be considered of usual occurrence and undoubtedly if more colonies had been examined it would have disclosed that more than one Campylobacter spp. were simultaneously excreted by the same lamb.

"C. fecalis" was isolated from 24.1 per cent faeces of ewes and only 2.5 per cent faeces of lambs showing that this species is more common in adult than in young animals. On the other hand, jejuni/coli GC was isolated from 24 per cent faeces of lambs and only 6.9 per cent faeces of ewes showing that these campylobacters are more frequently excreted by young stock. Generally, infections by C. coli in lambs occurred earlier than C. jejuni (Table 4.5). Consecutive infections where lambs acquired "C. fecalis", C. jejuni and C. coli indicated that previous exposure to one Campylobacter spp. did not prevent subsequent infection with other species.

Two lambs had suffered diarrhoea due to K99<sup>+</sup>EC and no campylobacters were isolated; the presence of Campylobacter spp. in other lambs or sheep was not associated with any clinical diarrhoea. Normal milk fed lambs have soft faeces it was not therefore possible to appreciate any slight alteration of the faecal consistency which could have been related with the excretion of some Campylobacter spp.

### 3) Ovine diagnostic material survey

Ovine faeces, rectal swabs and intestinal contents which had been sent for routine bacteriological diagnosis to the Veterinary Field Station, University of Edinburgh from September 1981 to October 1982, were used to investigate the occurrence of Campylobacter spp. Part of this material was used to compare selective media (Chapter 3, Section B).

#### Materials and Methods

Twenty faeces, 9 rectal swabs and 4 intestinal contents from 33 sheep were cultivated on PN according to the described first year procedure. The material was heterogenous in origin, most of the faeces had been taken from scouring sheep but some were from non-diarrhoeic animals suffering from other illness or from flocks in which diarrhoea was present. The age of animals ranged from 1 week old to old sheep.

Bacteriological works other than campylobacter isolations were done by Miss P. Wooding, University of Edinburgh.

Yates' corrected Chi-square was used (Downie and Heath, 1974) in statistical analysis of results.

#### Results

Campylobacters were isolated from 8 out of 33 (24.2 per cent) ovine faecal samples. Only one Campylobacter spp. was examined per animal. C. jejuni biotype 1 was isolated from 6 ovines, C. coli from one and untyped (lost due to contamination) Campylobacter spp. from another. These campylobacters were isolated from diarrhoeic animals with no other detected bacterial diseases. The distribution of these campylobacter positive animals according to their age is shown in Table 4.6. Similar isolation rates were found in 1 to 8-week-old

lambs and mature (1-year-old) sheep. Campylobacters were isolated from 25 per cent of the sheep and from 23 per cent of the lambs.

Chi-square comparisons between these campylobacter isolations from faeces (3/20) or swabs (5/9) were not significant (Yates' correction  $\chi^2 = 3.282$ ,  $p < 0.10$ ). Campylobacters were not isolated from the 4 intestinal contents.

Six 1-2 and one 3-week-old lamb also excreted E. coli considered to be possibly pathogenic. Other bacterial enteric pathogens were not incriminated other than in the case of two sheep of more than 1 year suffering from Johne's disease (Table 4.6).

Yates' corrected Chi-square comparisons between scouring and non-scouring campylobacter positive and negative sheep detected no significant differences, neither for the 8 campylobacter positive faeces ( $\chi^2 = 2.353$ ,  $p < 0.20$ ) nor for the 6 C. jejuni biotype 1 positive faeces ( $\chi^2 = 1.326$ ,  $p < 0.30$ ).

### Discussion

Campylobacters were isolated from 8 out of 24 diarrhoeic animals and this compared with 9 non-diarrhoeic animals in which campylobacters were not isolated. It is evident that this work provides insufficient non-diarrhoeic animals for comparison. Although these results are not significant, the recovery of campylobacters is much higher than recorded by other authors.

Firehammer and Myers (1981) were unable to isolate campylobacters from 36 diarrhoeic and from 20 healthy lambs. Prescott and Bruin-Mosch (1981) isolated jejuni/coli GC from 13.6 per cent healthy sheep whereas only 4.3 per cent were recovered from diarrhoeic animals. Thus it can be appreciated that isolation rates may vary greatly in



sheep and although no obvious association between diarrhoea and campylobacter recovery has been made the possibility of such a relationship cannot be excluded on the evidence available. These surveys have been too small, a problem exacerbated by the difficulty of obtaining good untreated samples from diarrhoeic sheep.

Interestingly, more campylobacters have been isolated from faecal swabs than from faeces. PN plates lightly inoculated with swabs gave more campylobacters than the heavily inoculated faecal PN plates. This observation should be given consideration because although the difference is not significant the number of samples studied is likely to have excluded a definitive conclusion. Rectal swabs collect material from the para-mucosal area of the rectal walls rather than faecal luminal contents. Campylobacters could be more numerous in the rectal ovine para-mucosal area than in faeces as has been demonstrated by comparative counts in a gnotobiotic calf (vide infra, Chapter 6, Section A). Besides, para-mucosal material probably contains more mucus than faeces and may protect campylobacters from desiccation. This latter reason could explain why campylobacters are able to survive in desiccated faecal swabs (Ullmann and Kischkel, 1981).



### General Discussion

Russell (1955) isolated a microaerophilic Vibrio spp. (Campylobacter spp.) from intestinal contents of hoggets suffering from profuse watery scouring and unsuccessfully tried to demonstrate its role as causative agent. Since then other authors have failed to demonstrate a correlation between the isolation of campylobacters and diarrhoea in sheep. In the 3 surveys undertaken it was not possible to demonstrate this correlation and it was only found that C. jejuni may be related to the presence of soft faeces without clinical diarrhoea in some susceptible, possibly non-immune sheep. In order to assess whether campylobacters are associated with clinical diarrhoea the data of these 3 surveys was pooled and analysed.

Chi-square comparison between the campylobacter isolation rate of 127 non-diarrhoeic animals from the 3 pooled surveys and that of 24 diarrhoeic animals from the diagnostic material survey failed to detect any significant difference (Table 4.7A). It could be argued that the campylobacter isolation rate of the non-diarrhoeic group is artificially increased due to the inclusion of the lamb survey<sup>in</sup> which animals had been sampled six times. When these 16 lambs were omitted the isolation rate of diarrhoeic sheep was still not significantly different from the non-diarrhoeic animals (Table 4.8). This analysis failed to demonstrate a link between the clinical enteric disease and the isolation of campylobacters although it has to be reemphasized that this conclusion is limited by the small number of diarrhoeic sheep available.

When the isolation of Campylobacter sp. from diarrhoeic and non-diarrhoeic animals were separately compared by Yates' corrected Chi-

square tests it was not possible to find any significant difference between these two groups of animals, neither for the slight increased isolation of C. jejuni in diarrhoeic animals ( $x^2 = 1.597$ ,  $p < 0.30 > 0.20$ ) nor for the presence of "C. fecalis" only in the non-diarrhoeic ovine group ( $x^2 = 1.966$ ,  $p < 0.20 > 0.10$ ) (Table 4.7B).

It is interesting to note that no CHI or C. fetus strains were isolated from any of the 151 animals examined despite 85 of the samples being cultivated on S or SN. Skirrow's medium is suitable for the isolation of both CHI and C. fetus and although Preston medium is more inhibitory for these microorganisms it nevertheless has allowed the frequent isolation of CHI and the occasional strain of C. fetus from bovine faeces (this chapter, Section B). On the other hand, "C. fecalis" which is highly inhibited by Preston and to a lesser extent also by Skirrow's medium, was isolated from 15 sheep showing that this campylobacter group is present in high numbers and are probably very much more widespread in the faecal flora than the isolation rates reflected by this work.

Florent (1959b) stated that Vibrio fetus (C. fetus) behaved in the ovine intestine as a saprophytic microorganism when he managed to re-isolate it from orally infected animals. El Azhary (1968) isolated V. fetus subsp. intestinalis from 10.6 per cent ovine faeces from a slaughterhouse. Recently, Roberts, Allan, Walker and Sommerville (1983) pointed out that C. jejuni is very common in the sheep population whereas C. fetus subsp. fetus is less frequently recovered. It is surprising that C. fetus strains have not been isolated from any of the animals of these 3 surveys although this result is in agreement with earlier observations of Firehammer (1965) who failed to isolate C.

fetus, even from faeces of ewes from naturally exposed flocks which formerly had suffered "vibriosis" outbreaks (abortions) whereas he promptly obtained "C. fecalis" from many of these animals. Firehammer's methods were suitable for the isolation of C. fetus as, following the same methodology, he was able to recover this bacterium from intra-ruminally infected ewes for as long as 25 days PI. Similarly, Smibert (1978) stated that he was unable to isolate C. fetus from sheep faeces although the filtration method he used may have been responsible. The results obtained by the present author show that although the presence of C. fetus cannot be excluded completely in the animals examined it is evident that this microorganism was not frequently excreted and it may well be that natural non-infected sheep flocks are more common than bovine herds. On the other hand, CHI strains were not isolated and their existence in sheep remains unproven.

CHAPTER 4: SECTION BBOVINE SURVEYS

These surveys were carried out in order to assess the campylobacter isolation rates in different cattle herds and to examine the relationship, if any, between the excretion of different Campylobacter spp. and diarrhoea. Some of the samples examined were from animals of heterogenous origin sent for diagnosis but the majority of the samples studied were from diarrhoeic and some non-diarrhoeic young calves. The relationship of campylobacters both with enteropathogens such as rotavirus, coronavirus, K99<sup>+</sup>EC in mixed infections of young calves and their possible involvement in enteric disease will be assessed. The Campylobacter spp. isolated provided strains for comparative taxonomic studies with bovine and ovine faecal strains.

Laboratory methodology was also evaluated in the following areas: comparison of procedures, influence of storage on isolation rates and, recovery from faecal swabs or faeces. The influence of these factors on the material examined and the interpretation of the results according to variations caused by them will be assessed.

An examination was made of the Campylobacter spp. excretion rates in beef and dairy calves and the effect of age on excretion. Limited serotyping of a few C. jejuni and C. coli strains was also arranged in order to provide epidemiological information and fully characterize the strains which were used later in animal experiments.

1) Bovine diagnostic material survey

Bovine faeces, rectal swabs and intestinal contents which had been sent for routine bacteriological diagnosis to the Veterinary

Field Station, University of Edinburgh from September 1981 to October 1982 were used to investigate the incidence of Campylobacter spp. Some of this material had been used in a comparison of selective media (Chapter 3, Section B).

### Materials and Methods

Sixty one faeces, 51 rectal swabs and 2 intestinal contents from 114 bovines were cultivated on PN according to the already described first year procedure. The material was heterogeneous, from diarrhoeic and non-diarrhoeic animals of different ages, breeds, origin and clinical status. The animals belonged to a variety of breeds (Table 4.11) with ages as follows: 32 calves of 1-2 weeks, 26 calves of 3-4 weeks, 6 calves of 5-8 weeks, 18 animals of 9 weeks to 1 year and 32 animals of more than 1 year (Table 4.9). Single samples were obtained from animals in 88 different herds whilst multiple samples (2-4) were derived from 11 additional premises.

Eighty five animals had diarrhoea or other faecal alteration such as soft bloody or mucoid faeces and they were considered as a diarrhoeic bovine group (Table 4.9). Another 29 animals had no symptoms of faecal alteration and they were pooled as non-diarrhoeic bovine group. This diseased non-diarrhoeic group is composed of animals from which samples had been submitted because of different clinical conditions: 11 animals with pneumonia, 3 with pyrexia, 2 with cough, 1 with chronic weigh loss, 1 with suspect ulcer, 1 with ruptured pharynx, 2 with mastitis, 1 with actinomycosis, 1 with heart disease and emphysema, 1 losing hair and enlarged MLN, 1 with intussusception, 2 were clinically described as dull and 2 were without specific symptoms.

Statistical comparisons were made by Yates' corrected Chi-square

(Downie and Heath, 1974) and Brandt and Snedecor method (Snedecor, 1950).

Most of the samples had been taken by the large animal practice of the Veterinary school, some came from the Veterinary Field Station hospital. Bacteriological work other than campylobacter isolations were carried out by Miss P. Wooding, University of Edinburgh.

### Results

Campylobacters were isolated from 47 out of 114 (41.2 per cent) bovine faecal samples. Overall 27.2 per cent animals excreted jejuni/coli GC and 12.3 per cent fetus GC. Only one Campylobacter sp. was isolated per animal in 46 animals. Two types of colonies were detected on a PN plate of a 1-year-old heifer suffering from diarrhoea and they were identified as C. coli and CHI type 1. Forty eight campylobacter strains were identified as follows: 15 C. jejuni biotype 1, 1 C. jejuni biotype 2, 15 C. coli, 7 CHI type 1, 4 CHI type 2, 3 C. fetus and 3 unclassified campylobacters detected in carbol-fuchsin smears and not isolated due to contamination of the CBA subculture plates.

Campylobacters were isolated from 39 out of 85 (45.9 per cent) animals of the diarrhoeic group and from 8 out of 29 (27.6 per cent) animals of the diseased non-diarrhoeic group (Table 4.9). These differences were not significant either for the uncorrected Chi-square ( $\chi^2 = 2.987$ ,  $p < 0.10 > 0.05$ ) or for the Yates' corrected Chi-square ( $\chi^2 = 2.280$ ,  $p < 0.20 > 0.10$ ).

All the Campylobacter spp. were isolated from both diarrhoeic and non-diarrhoeic animals (Table 4.10). When jejuni/coli GC and fetus GC strains were pooled without considering the untyped strains, no

significant differences were found in their distribution between diarrhoeic and non-diarrhoeic animals. From 38 campylobacter strains isolated from the diarrhoeic group of animals 27 (71.1 per cent) belonged to the jejuni/coli GC and 11 (28.9 per cent) to the fetus GC. From 7 strains isolated from the non-diarrhoeic group 4 belonged to the jejuni/coli GC and 3 to the fetus GC (Yates' corrected  $\chi^2 = 0.820$ ,  $p < 0.50 > 0.30$ ).

The total campylobacter isolation rates of the 5 age groups is shown in Table 4.9. No significant differences were found among the campylobacter isolation rates of different age groups by the Brandt and Snedecor Chi-square method either for the diarrhoeic animals ( $\chi^2_{(4)} = 4.726$ ,  $p < 0.50 > 0.30$ ), the non-diarrhoeic animals ( $\chi^2_{(4)} = 4.944$ ,  $p < 0.30 > 0.20$ ) or both groups considered together ( $\chi^2_{(4)} = 7.236$ ,  $p < 0.20 > 0.10$ ).

No significant differences were found among the campylobacter isolation rates for different cattle breeds by the Brandt and Snedecor Chi-square method ( $\chi^2_{(5)} = 4.195$ ,  $p < 0.70 > 0.50$ ) (Table 4.11). When the data was pooled as dairy or beef cattle it was found that campylobacters were isolated from 17 out of 47 (36.2 per cent) dairy cattle and from 27 out of 55 (49.1 per cent) beef cattle. This isolation rate is not significantly different ( $\chi^2 = 1.238$ ,  $p < 0.30 > 0.20$ ).

Campylobacters were isolated from 23 out of 61 (37.7 per cent) faeces, 23 out of 51 (45.1 per cent) rectal swabs and from 1 of 2 intestinal contents. Similar isolation rates were obtained from faeces or rectal swabs and no significant differences were detected ( $\chi^2 = 0.627$ ,  $p < 0.50 > 0.30$ ). Seventeen jejuni/coli GC, 5 fetus GC and 1 untyped strain were isolated from rectal swabs. Fourteen jejuni/



coli GC, 8 fetus GC and 2 untyped strains were isolated from faeces. CHI type 2 was isolated from a small intestinal contents. Two strains, C. coli and CHI type 1, were isolated from one faeces. The isolation rate of jejuni/coli GC and fetus GC strains from faeces or swabs was not significantly different (Yates' corrected  $\chi^2 = 1.747$ ,  $p < 0.20 > 0.10$ ).

Infection with campylobacters and other bacteria were as follows: Salmonella typhimurium/C. jejuni biotype 1 from one 1-month-old calf, E. coli (possibly enteropathogenic)/C. coli from 2 calves of less than 1 week of life and Mycobacterium paratuberculosis/C. jejuni biotype 1 from one adult animal. Other enteric infections not involving campylobacters were: S. typhimurium from a 4-week-old calf, S. typhimurium/M. paratuberculosis from two adult cattle and S. typhimurium/S. virchow from one adult animal.

### Discussion

More campylobacters were isolated from the diarrhoeic group of cattle but this difference was found not to be statistically significant. When the data was analysed separately it was found that campylobacters were excreted by either the diarrhoeic or non-diarrhoeic bovine groups whereas other bacterial agents, such as Salmonella spp., Escherichia coli or Mycobacterium spp., had been diagnosed only in diarrhoeic animals. The analysis of the results of this survey failed to demonstrate any link between clinical diarrhoea and the faecal isolation rate either of all campylobacters or of one particular species of campylobacter. These findings confirm those observations in which campylobacters were isolated from non-diarrhoeic healthy carrier cows (El Azhary, 1968; Robinson, 1982; Robinson and Jones, 1981; Doyle and Roman, 1982; Oosterom, Engels, Peters and Pot, 1982;



Elegbe, 1983), heifers (Clark, Monsborough and Dufty, 1969) or calves (El Azhary, 1968). Lederle (1963), Al-Mashat and Taylor (1980a) and Firehammer and Myers (1981) isolated campylobacters from diarrhoeic calves but they did not compare the results with a representative number of non-diarrhoeic animals. Interestingly, Firehammer and Myers (1981) isolated campylobacters from the only 3 non-diarrhoeic controls which they had selected at random from the affected farms. Allsup and Hunter (1973) isolated significantly more campylobacters from a "diseased" group of calves than from a "healthy" one, but it has to be pointed out that they were not comparing diarrhoeic with non-diarrhoeic calves but groups and within the "diseased" group only 9 out of 36 calves had had enteric disorders and the rest were animals with other diseases comparable to the non-diarrhoeic group of this work. Prescott and Bruin-Mosch (1981) obtained similar campylobacter isolation rates from healthy or diarrhoeic calves and steers.

Two Campylobacter spp. were isolated from the same cow because jejuni/coli GC and fetus GC colonies were detected at the first subculture on PN plates. Probably if the second year procedure had been used more mixed infections would have been detected.

Campylobacters were isolated from 26 out of 64 animals (40.6 per cent) of 1 to 8 weeks of age and from 21 out of 50 animals (42 per cent) of more than 9 weeks of age. No significantly different campylobacter isolation rates could be demonstrated between the age groups. Nevertheless, El Azhary (1968) found that the number of fetus GC strains isolated from the faeces of adult non-diarrhoeic cows were lower than that obtained from 2-month-old non-diarrhoeic calves. Florent (1959b) and Al-Mashat and Taylor (1980a) working

with a small number of samples found the same decrease in the isolation rate of older cattle. El Azhary (1968) supposed that this could be due to acquired immunity resulting in a lower population or even the disappearance of the campylobacters from the intestinal tract. Robinson (1982) cultivating fortnightly rectal swabs of adult dairy non-diarrhoeic cows found that the excretion of jejuni/coli GC was low and intermittent. It may well be that the bacteriological techniques employed in this work were more sensitive than those used by Florent (1959b), El Azhary (1968) and Al-Mashat and Taylor (1980a), being capable of detecting low numbers of bacteria and therefore improving considerably the isolation rate from older animals. If this is true it has to be supposed that the number of campylobacters decreases considerably rather than disappearing and adult cattle may be the permanent source of infection of younger stock.

Similar isolation rates were obtained from faeces or rectal swabs. Although only a non-significant increase in the campylobacters isolated from swabs was demonstrated it should be mentioned that the amount of faeces cultivated in these swabs was in general small. The reason for this was that the one swab was previously inoculated on CBA and MacConkey for diagnostic purposes and always, to avoid antibiotic inhibition of other bacteria, the last plate to be cultivated with the remaining material was the PN plate. Despite this the isolation performance of rectal swabs was comparable to that of PN plates heavily inoculated with faeces.

## 2) A.D.R.A. survey

Acute neonatal calf diarrhoea is responsible for major economic losses in many dairy and beef herds. Although many infectious agents have been implicated in the aetiology of this disease, four micro-organisms stand out as being of widespread occurrence and proved enteropathogenicity, rotavirus, coronavirus, ETEC and Cryptosporidium sp. (Morin, Larivière and Lallier, 1976; Tzipori, 1981; Sherwood, 1982).

Control of the disease due to rotavirus and ETEC can be attempted through dam vaccination to elevate the titers of specific antibody ingested by the calf in colostrum and milk. Some of the samples examined in this survey were obtained from herds in which experimental vaccines were under test. During two consecutive years half of the cow population of some farms was vaccinated with vaccines No. 1 or No. 2. Both vaccines contain rotavirus and K99 pilus antigens as described by Snodgrass, Nagy, Sherwood and Campbell (1982). Vaccine No. 2 contains an increased concentration of rotavirus antigen and according to preliminary studies has produced a better serological response than vaccine No. 1 (Snodgrass, Personal Communication, 1984).

The association of campylobacters with neonatal diarrhoea has been suggested (Allsup, Matthews, Hogg and Hunter, 1972; Allsup and Hunter, 1973; Al-Mashat and Taylor, 1980a; Firehammer and Myers, 1981) but not proved as these bacteria can also be isolated from apparently healthy calves (Florent, 1959b; El Azhary, 1968; Prescott and Bruin-Mosch, 1981). When this study was undertaken no surveys had been carried out to ascertain the campylobacter excretion rates of diarrhoeic and non-diarrhoeic young calves. Recently, two preliminary

reports have been published on the campylobacter isolation rates in diarrhoeic and non-diarrhoeic calves (Morgan, Hall, Reynolds and Parsons, 1983) and a long term study on the excretion rates of calves from a beef suckler herd (Roberts, Allan, Walker and Sommerville, 1983). Up till the present time no published work has tried to assess the possible significance of campylobacters in relation to the presence of other pathogens in diarrhoeic calves.

Arrangements had been made whereby calf faeces obtained from herds experiencing considerable clinical diarrhoeal disease were submitted directly or indirectly to the A.D.R.A. Institute. This was part of a continuing investigation into the cause and control of neonatal ruminant diarrhoea. Selected samples which had been submitted between November 1981 and April 1983 were used for this study. Some of the samples were also used to compare selective media and to evaluate the survival of campylobacters in calf faeces (Chapter 3, Sections C and D).

The main purpose of this part of the work was to assess the faecal excretion rate of different Campylobacter spp. in diarrhoeic and apparently non-diarrhoeic calves from herds with outbreaks of neonatal diarrhoea in which the presence of other enteropathogens was also ascertained. It was hoped that such a study might more clearly define the possible role of campylobacters in neonatal diarrhoea or possible associations with particular enteropathogens. There was the additional expectation that if the experimental vaccines proved satisfactory in suppressing the accepted pathogenicity of K99<sup>+</sup>EC and/or rotavirus that such protected animals might more clearly demonstrate the pathogenicity of less virulent pathogens such as

the campylobacters.

A limited number of jejuni/coli GC strains from 4 farms were serologically classified for epidemiological purposes. Different procedures for isolation and biotyping were used during each of the two years of the survey and additional information was obtained about the usefulness of these laboratory techniques.

#### Materials and Methods

Faecal samples were collected as indicated in Chapter 2 from calves of less than 1 month old and subsequently stored as described in Chapter 3, Section D. Most of the samples were taken from calves that had recently become diarrhoeic and before treatment was initiated, but also a few faeces were collected after treatment. Lesser numbers of clinically non-diarrhoeic calves from some of the same herds suffering from neonatal diarrhoea were chosen at random as controls. Whenever possible, the following information was recorded: beef suckler or dairy herd, vaccination trial herd with rotavirus-ETEC vaccine 1 or 2 and diarrhoeic, non-diarrhoeic or treated animal.

Overall 687 calf faeces from 85 outbreaks of neonatal diarrhoea derived from 80 farms were studied. In 5 farms, clinical disease appeared and samples were obtained in each year. Each faecal sample corresponded to a different animal. Faeces were cultivated on PN plates according to the first and second year procedures. Three hundred and seventy one faeces from 55 outbreaks were cultivated since the 2nd of December 1981 until the 28th of October 1982 following the first year procedure and 316 faeces from 30 outbreaks according to the second year technique since the 19th of November 1982 until the 29th of April 1983. As described in Chapter 3,

Section C, part of this survey was used to compare PN and RNBGT media, but in this chapter for comparative purposes the campylobacters which were isolated by means of PN plates only were considered, those obtained from the corresponding RNBGT plate were disregarded.

Examination for enteropathogens other than campylobacters was carried out at A.D.R.A., Moredun Institute as described in Chapter 2.

Passive haemagglutination test (PHA) for jejuni/coli GC was performed by Dr. L. Roberts in the North of Scotland College of Agriculture, Veterinary Investigation Division, Aberdeen, according to Penner and Hennessy (1980).

Statistical comparisons were made by Yates' corrected Chi-square tests (Downie and Heath, 1974).

- Division into groups on the basis of vaccination and sample procedure

As the samplings of the calves from herds experimentally immunised with types 1 or 2 vaccine coincided with the use of the first and second year campylobacter procedures respectively, the calf neonatal diarrhoea outbreaks were divided into the following 4 groups:

GROUP A: Farms not using vaccine from which faecal calf campylobacter excretion was investigated by the first year procedure.

GROUP B: Farms not using vaccine from which faecal campylobacter excretion was investigated by the second year procedure.

GROUP C: Farms in which half of the cows had been inoculated with type 1 vaccine and faecal calf campylobacter excretion was investigated by the first year procedure.

GROUP D: Farms in which half of the cows had been inoculated with type 2 vaccine and faecal calf campylobacter excretion was investigated

by the second year procedure.

## Results

### 1.- Calf enteropathogen excretion rates from neonatal diarrhoea groups

1.1 General enteropathogen excretion rates: All calf samples considered in this study were investigated for campylobacters and many of them were examined for other enteropathogens, nearly all for rotavirus and cryptosporidia, many of them for coronavirus and nearly two-thirds for K99<sup>+</sup>EC. Overall 310 out of 687 calves (45.1 per cent) excreted campylobacters, 223 out of 680 (32.8 per cent) cryptosporidia, 160 out of 680 (23.5 per cent) rotavirus, 48 out of 632 (7.6 per cent) coronavirus and 12 out of 452 (2.6 per cent) K99<sup>+</sup>EC (Table 4.12A).

The enteropathogen excretion rates for the aforementioned 4 groups are summarized in Table 4.12A. Inspection of the data shows that calves from group D farms excreted less rotavirus and more cryptosporidia and campylobacters than any of the other groups. Yates' corrected Chi-square comparisons between group D calves and the rest disclosed highly significant differences either for the lower isolation rate of rotavirus ( $\chi^2 = 48.916$ ,  $p < 0.001$ ) or for the higher ones of cryptosporidia ( $\chi^2 = 48.622$ ,  $p < 0.001$ ) and campylobacters ( $\chi^2 = 33.895$ ,  $p < 0.001$ ).

1.2 Campylobacter spp. excretion rates: The Campylobacter spp. isolated throughout the survey are shown in Table 4.12B. C. jejuni was isolated from 23.6 per cent of the calves examined and was the most frequently isolated species in all four groups. CHI was after C. jejuni the most common isolate (14.8 per cent). C. coli and C. fetus were obtained from 5.7 per cent and 3.2 per cent of the calves



respectively. Campylobacters were also detected by carbol-fuchsin smears on heavily contaminated CBA subculture plates of 16 animals (2.3 per cent) but they were not isolated and biotyping tests could not be carried out.

As referred to before group D calves had a significantly increased isolation rate of campylobacters. When the species were pooled as jejuni/coli GC and fetus GC and compared it became clear that this calf group had a significantly higher isolation of fetus GC which was mainly the cause of the increment (Table 4.12B,  $x^2 = 54.622$ ,  $p < 0.001$ ).

## 2.- Excretion of campylobacters in dairy or suckler beef calves

Table 4.13 shows the enteropathogen excretion rates of groups A, B, C and D subdivided into beef or dairy type of herds. Where the type of herd was uncertain the herd was classified as unknown. Observing the beef or dairy campylobacter isolation rates in the 4 groups it is noted that calves from beef herds have in all cases a higher percentage of isolation than dairy cows. When these data were pooled (Table 4.14) a significantly higher campylobacter excretion rate in calves from beef was found than in animals from dairy herds ( $x^2 = 31.141$ ,  $p < 0.001$ ) whereas the isolation rate for any of the other enteropathogens is very similar in both types of herds.

The relationship of different Campylobacter spp. with this phenomenon was analysed. When jejuni/coli GC and fetus GC were compared to each other - without including the animals of unknown origin - it was discovered that dairy calves had a significantly lower isolation rate of jejuni/coli GC than beef animals whereas



fetus GC isolation rates were very similar in both types of calves (Table 4.15A,  $x^2 = 56.629$ ,  $p < 0.001$ ). Table 4.15B shows the isolation rates of individual Campylobacter spp. and clearly, dairy herds have significantly reduced C. jejuni ( $x^2 = 42.914$ ,  $p < 0.001$ ) and also to a lesser extent, a decreased C. coli excretion ( $x^2 = 6.568$ ,  $p < 0.02 > 0.01$ ), whereas the isolation rates of CHI type 2 and C. fetus are nearly the same and CHI type 1 was slightly increased in dairy calves. In order to eliminate distortion of the results due to a possible differential antibiotic treatment which could have been given to either type of herds, all animals without any clinical data or those which had been treated, with or without isolation of campylobacters, were excluded (Table 4.15C) and when Yates' corrected Chi-square was performed a significant difference was also found either for C. jejuni ( $x^2 = 44.013$ ,  $p < 0.001$ ) or C. coli ( $x^2 = 6.674$ ,  $p < 0.01 > 0.001$ ).

### 3.- Examination of associations between diarrhoea and excretion of enteropathogens

All group C and D calves studied were diarrhoeic but in groups A and B some non-diarrhoeic animals had been chosen at random from the diseased herds. Animals of unknown clinical state or treated were considered separately either for group A (Table 4.16A) or B (Table 4.16B) and were not included when the overall data of these two groups were pooled (Table 4.17).

3.1 Campylobacter excretion: Interestingly, non-diarrhoeic calves from group A or pooled A and B excreted more campylobacters than diarrhoeic animals of the same groups and these differences were found significant at the 2 or 5 per cent levels respectively by

Yates' corrected Chi-square (Table 4.16A,  $x^2 = 6.379$ ,  $p < 0.02 > 0.01$  and Table 4.17,  $x^2 = 4.516$ ,  $p < 0.05 > 0.02$ ). In group B1 also non-diarrhoeic animals excreted more campylobacters but this difference considered separately was found not significant (Table 4.16B,  $x^2 = 0.071$ ,  $p < 0.80 > 0.70$ ).

In Table 4.18 the isolation rate of jejuni/coli GC and fetus GC of diarrhoeic and non-diarrhoeic animals is compared. In group A non-diarrhoeic calves excreted 11.8 or 7 per cent more campylobacters of jejuni/coli or fetus groups respectively than the diarrhoeic animals. When the data of the groups was pooled no significant differences were obtained for the isolation rates of diarrhoeic and non-diarrhoeic calves either for jejuni/coli GC ( $x^2 = 0.041$ ,  $p < 0.90 > 0.80$ ) or for fetus GC ( $x^2 = 0.391$ ,  $p < 0.70 > 0.50$ ).

**3.2 Other enteropathogens:** More diarrhoeic animals of group A excreted rotavirus than non-diarrhoeic ones and this difference was found to be statistically significant (Table 4.16A,  $x^2 = 14.040$ ,  $p < 0.001$ ). The few diarrhoeic and non-diarrhoeic calves studied in group B had the same rotavirus excretion rate (Table 4.16B) but when the data of both groups (A and B) were pooled the increased rotavirus excretion rate of diarrhoeic calves was also significant (Table 4.17,  $x^2 = 10.451$ ,  $p < 0.01 > 0.001$ ).

A slightly increased coronavirus isolation rate of diarrhoeic animals of group A was found to be not significantly different from the one of non-diarrhoeic calves of this group (Table 4.15,  $x^2 = 0.144$ ,  $p < 0.80 > 0.70$ ). Coronavirus was not detected in the faeces of any diarrhoeic or non-diarrhoeic group B calves. Coronavirus isolation rates of diarrhoeic calves of groups C and D are similar

to those of group A (Table 4.12A).

An increased cryptosporidium demonstration rate of calves from groups A1, pooled A, B1 or pooled A and B was found not to be significantly different from that of non-diarrhoeic animals by Yates' corrected Chi-square (Tables 4.16A,  $x^2 = 1.718$ ,  $p < 0.20 > 0.10$  and  $x^2 = 1.995$ ,  $p < 0.20 > 0.10$ ; 4.16B,  $x^2 = 0.054$ ,  $p < 0.90 > 0.80$  and 4.17,  $x^2 = 2.204$ ,  $p < 0.20 > 0.10$  respectively). The cryptosporidial excretion of diarrhoeic calves of group C (Table 4.12A, 25.4 per cent) was very similar to that of diarrhoeic calves of pooled groups A and B (Table 4.17, 26.4 per cent). In contrast to this, the significantly higher isolation rate of the diarrhoeic animals from group D (Table 4.12A, 50 per cent) clearly shows that diarrhoea in this group of animals could be due to the parasitism.

Only 12 out of 181 (6.6 per cent) diarrhoeic non-vaccinated group of calves excreted K99<sup>+</sup>EC indicating that this bacterium was not a frequent cause of diarrhoea. None of 32 non-diarrhoeic calves excreted K99<sup>+</sup>EC (Table 4.17).

#### 4.- Effect of vaccination on enteropathogen excretion

The cows in herds of groups C and D had been respectively inoculated with vaccines of type 1 or 2. Both vaccines may have effectively prevented the infection of K99<sup>+</sup>EC as no isolations were made in any of 206 diarrhoeic vaccinated calves examined (Table 5.12A).

The protection conferred by the two types of vaccines on rotavirus infection differed. Diarrhoeic calves from group C in which cows were injected with type 1 vaccine had a similar rotavirus isolation rate (4.12A, 30 per cent) to calves from unvaccinated cows pooled groups A and B (Table 4.17, 39.8 per cent). On the

other hand, the diarrhoeic calves from the vaccinated group D in which cows were inoculated with type 2 vaccine had statistically a significantly diminished excretion rate of rotavirus (Table 4.12A, 7.9 per cent).

Vaccinated diarrhoeic calves of groups C and D excreted more campylobacters than diarrhoeic animals of non-vaccinated groups A and B. In fact, 201 out of 370 (54.3 per cent) (Table 4.12A) diarrhoeic calves of vaccinated groups C and D excreted campylobacters in contrast with only 76 out of 238 (31.9 per cent) (Table 4.17) diarrhoeic non-vaccinated calf groups A and B, these differences are statistically highly significant by Yates' corrected Chi-square test ( $\chi^2 = 28.382$ ,  $p < 0.001$ ).

Interestingly, group D in which rotavirus, the major cause of diarrhoea in most outbreaks had been considerably reduced, had a significantly increased excretion of both cryptosporidia and campylobacters (Table 4.12A).

#### 5.- Effect of laboratory procedures and sampling on campylobacter isolation rates

5.1 Difference between first and second year procedures: Observing Table 4.12A it is evident that more campylobacters were recovered by the second year procedure (groups B and D) than were obtained by the first year technique (groups A and C). In fact, considering the non-vaccinated farms, campylobacters were isolated from 48.7 per cent group B calves whereas group A yielded only 29.9 per cent ( $\chi^2 = 8.245$ ,  $p < 0.01 > 0.001$ ). Similarly, in the vaccinated farms campylobacters were recovered from 60.4 per cent group D calves whereas only 43.1 per cent of group C were found to excrete

( $x^2 = 9.531$ ,  $p < 0.01 > 0.001$ ). When these two-year-survey data were pooled it was found that in the first year group 128 out of 371 calves (34.5 per cent) excreted campylobacters whereas using the second year methodology 182 out of 316 calves (57.6 per cent) were found to be positive for these bacteria, these differences being statistically significant by Yates' corrected Chi-square test ( $x^2 = 35.827$ ,  $p < 0.001$ ).

By means of the first year procedure 3 animals out of 371 (0.8 per cent) yielded two Campylobacter spp. per calf whereas using the second year technique the double isolations were increased to 28 out of 316 (8.9 per cent) (Tables 4.12B and 4.19). The use of the second year procedure allowed the isolation of significantly more jejuni/coli GC ( $x^2 = 16.370$ ,  $p < 0.001$ ) and fetus GC ( $x^2 = 49.824$ ,  $p < 0.001$ ) although it clearly improved more the isolation of the latter group (Table 4.19).

5.2 Effect of age of sample: In Chapter 3, Section D, the period of time elapsed between the collection of samples at the farms and their cultivation was calculated for 554 out of the 687 (80.6 per cent) survey faeces and a statistically significant decline of the faecal isolation rates from the 7th day of storage onwards was found. For that reason it was considered necessary to classify these faeces into the following two groups: stored up to 6 days inclusive ("fresh faeces") and from 7 days onwards ("old faeces"). Table 4.20A shows the effect of storage on the isolation rates from faeces. Overall 136 out of 236 (57.6 per cent) faeces from 51 outbreaks were positive for campylobacters when stored for 6 or less days whereas 130 out of 318 (40.8 per cent) faeces from

48 outbreaks were positive for campylobacters when stored for 7 days or more. Faeces of both storage periods were present unevenly distributed in 24 of the outbreaks whereas the remaining 51 outbreaks contained either "fresh faeces" in 27 outbreaks, or only "old faeces" in other 24 outbreaks. It can be observed in Table 4.20A that fewer campylobacter isolations were obtained from each of the outbreak sub-groups containing "old faeces", except for the vaccinated suckler beef herd C2 which contained very few animals.

The proportion of "fresh" and "old" faeces examined from calf suckler beef and dairy herds showed no significant differences (Table 4.20B,  $\chi^2 = 0.435$ ,  $p < 0.70 > 0.50$ ) indicating that age of samples was not responsible for the differences noted in these 2 groups.

The storage data of 313 out of 371 (84.4 per cent) faeces examined during the first year and of 241 out of 316 (76.3 per cent) studied during the second year were available. More than half of the faeces were "fresh" during the first year survey but only 32.4 per cent of the second year samples were "fresh". These differences were statistically significant (Table 4.20C,  $\chi^2 = 17.538$ ,  $p < 0.001$ ).

Interestingly, the group D compared with pooled outbreak groups A, B and C also had significantly less "fresh faeces" although this was the calf group from which most campylobacters were isolated (Table 4.20D,  $\chi^2 = 15.473$ ,  $p < 0.001$ ).

#### 6.- Herd outbreaks

In order to analyse the frequency and the different combinations of agents present in outbreaks the data of the outbreaks in which

5 or more calves were simultaneously examined for any of the 5 enteropathogens investigated were considered. Forty two outbreaks contained samples from more than 4 calves, comprising 86.7 per cent of the total calf samples examined. Details of the demonstration of enteropathogens in these 42 outbreaks classified according to the aforementioned group scheme, is shown in Tables 4.21. In 3 farms outbreaks were followed up in consecutive years, outbreaks identified as D531, D529-557 and D530 (Table 4.21F) occurred on the same farms as the ones identified as D3369, D3088 and D2725 (Table 4.21H) respectively. Two outbreaks, D128 and D338, were investigated by two campylobacter methodologies and for that reason they were separately considered in this study in groups A1 (Table 4.21A) and B1 (Table 4.21D).

It can be observed in Tables 4.21 that mixed infections are common in nearly all herd outbreaks. In fact, single agents were detected in only 4 outbreaks whereas calves from 17 herds simultaneously excreted 4 agents, 14 three agents and 7 two agents (Table 4.22A). In all these 42 diarrhoeic herds in which a minimum number of 5 animals was investigated, enteropathogens were detected. The three more frequent combinations were rotavirus, coronavirus, cryptosporidia and campylobacters in 15 herds, rotavirus, cryptosporidium and campylobacters in 11 herds and cryptosporidia and campylobacters in 4 herds. Single K99<sup>+</sup>EC infections were detected in two herds and calves of two other herds excreted only cryptosporidia or campylobacters. The herd enteropathogen incidence for each agent considered separately is shown in Table 4.22B; campylobacters were the most widespread agents as they were isolated from 88.1 per cent



of the herds.

Inspecting the data of Tables 4.21 it is evident that in many cases enteropathogens were detected in only a few animals. In order to make a more critical assessment of the possible importance of enteropathogens on farms, all enteropathogens isolated from 20 or less per cent of the calves were arbitrarily discounted (enteropathogen names in brackets in Tables 4.21). Following this analysis 9 herds had triple infections, 19 herds double, 11 herds single and 3 herds no enteropathogen was significantly excreted (Table 4.22A). The three more frequent agent combinations were rotavirus, cryptosporidium and campylobacters in 8 herds, cryptosporidium and campylobacters in 11 herds and rotavirus and campylobacters in 4 herds. Significant single infections were detected as follows: rotavirus 5 herds, K99<sup>+</sup>EC 2 herds, cryptosporidium 2 herds and campylobacters 2 herds. The separated herd incidence of each agent excreted by more than 20 per cent of the calves is considered in Table 4.22B. The 3 more widespread agents among herds were campylobacters (66.7 per cent), cryptosporidium (54.7 per cent) and rotavirus (45.2 per cent). Interestingly, the coronavirus herd frequency was reduced from 50 to 7.1 per cent when the 20 per cent significance level was introduced showing that this agent was common in half of the outbreaks of this study but only detected in few animals of each herd. On the other hand, K99<sup>+</sup>EC was significantly isolated from 3 out of the 4 positive herds showing that despite being not commonly found is nevertheless frequently isolated when detected in individual outbreaks.

C. jejuni was the most widespread Campylobacter spp. which



was isolated from 73.8 per cent of the herds and was excreted by more than 20 per cent of the animals in 38.1 per cent of the herds (Table 4.22C). Other Campylobacter spp. were isolated from 52.4 per cent (CHI type 1), 26.2 per cent (C. coli), 23.8 per cent (CHI type 2) and 19 per cent (C. fetus) of the herds. Only CHI type 1 and C. coli were isolated from more than 20 per cent of the animals in 11.9 and 7.1 per cent of the herds respectively (Table 4.22C).

C. jejuni was isolated from 82.8 and 50 per cent of the beef suckler and dairy herds respectively and was isolated from more than 20 per cent of the calves examined in 51.7 per cent of the beef suckler herds but in no dairy herd (Table 4.22D). C. coli was isolated from 31 per cent of the beef suckler herds and only from 8.3 per cent of the dairy herds and it was excreted by more than 20 per cent of the animals from 10.3 per cent of the beef suckler herds (Table 4.22D).

#### 7.- Enteropathogen isolation rates and Penner and Hennessy serotyping of some calves from 5 beef suckler herds

Table 4.23 detailing 5 beef suckler outbreaks is an example of the complexity of the data analysed here. In all these animals campylobacters were isolated either as single agents or in combination with other enteropathogens or different Campylobacter spp. Four campylobacter strains were chosen from 4 of the outbreaks to be used in infection experiments with gnotobiotic ruminants in Chapter 6. Penner and Hennessy's (1980) passive haemagglutination (PHA) scheme was used to serotype previously cloned jejuni/coli GC strains, one from each of 37 calves of 4 outbreaks. Twenty eight

C. jejuni strains from 3 different outbreaks B2464 (unvaccinated, first year), D523 (vaccinated, first year) and D3369 (vaccinated, second year) could be classified into 2 serological groups: 14 strains shared the antigen 2 and the other 14 the antigens 4, 16, 43, 50. Both serological groups are similarly distributed in the 3 outbreaks. Nine C. coli strains from outbreak D531 (vaccinated, first year) - which corresponds to the same farm as D3369 - were serotyped, 7 contained a distinct antigen 20 whereas two others shared antigens 4 and 43 with other C. jejuni strains.

8.- Interrelationships between the excretion of enteropathogens in calves from A.D.R.A. subgroups

Two by two Yates' Chi-square comparisons between the excretion of the following enteropathogens with each other were made: rotavirus, coronavirus, cryptosporidium, K99<sup>+</sup>EC, campylobacters in general and jejuni/coli GC and fetus GC in particular. Independent comparisons were made in each of the following subgroups: A1, A2, B1, B2, C1, C2, D1 and D2. From all these statistical tests only those with significance will be summarized here.

Calves from beef suckler subgroups B1, C1 and D1 were more likely to excrete at the same time campylobacters and cryptosporidia. The differences were just near significance for group B1 (Table 4.24A,  $x^2 = 3.443$ ,  $p = 0.0635$ ) and significant for groups C1 (Table 4.24B,  $x^2 = 6.517$ ,  $p < 0.02 > 0.01$ ) and D1 (Table 4.24D,  $x^2 = 3.906$ ,  $p < 0.05 > 0.02$ ). When the data was pooled (B1, C1, D1) a very significant difference was disclosed (Table 4.24E,  $x^2 = 20.196$ ,  $p < 0.001$ ). Only in subgroup C1 was it found that calves which excreted cryptosporidium had also a significantly increased jejuni/

coli GC isolation rate (Table 4.24C,  $x^2 = 6.578$ ,  $p < 0.02 > 0.01$ ).

Individual Chi-square comparisons of dairy subgroups (A2, B2, C2 and D2) did not show significant differences in any group.

Nevertheless when the data of all A.D.R.A. calves was pooled it was found that both jejuni / coli GC (Table 4.24F,  $x^2 = 7.195$ ,  $p < 0.01 > 0.001$ ) and fetus GC (Table 4.24G,  $x^2 = 25.880$ ,  $p < 0.001$ ) were isolated more often from animals infected with cryptosporidia than from calves without a detectable excretion of oocysts.

It was found that the few calves from subgroup A1 excreting K99<sup>+</sup>EC did not yield campylobacters probably because colibacillosis is a specific disease of very young calves which have not therefore had time to be infected with other bacteria (Table 4.25,  $x^2 = 3.657$ ,  $p = 0.0558$ ).

Finally, in subgroup C1 it was found that the few calves which excreted coronavirus generally did not harbour campylobacters (Table 4.26,  $x^2 = 4.832$ ,  $p < 0.05 > 0.02$ ).

### Discussion

Campylobacters were the most widespread of the enteropathogens as these microorganisms were isolated from 88 per cent of the outbreaks of more than 4 calves, and were excreted by more than 20 per cent of the animals in 66.7 per cent of these outbreaks. C. jejuni was the most common species, isolated from 73.8 per cent of the herds and excreted by 23.6 per cent of the calves examined. These bacteria also had a higher isolation rate than any of the other 4 enteropathogens studied in calf neonatal diarrhoea outbreak groups B, C and D and were present in most of the combined mixed infections in the farms. Cryptosporidium was after campylobacters

more widespread in the outbreaks than any of the other 3 agents and their overall isolation rate was higher than that of rotavirus due to a very significant excretion rate from both beef and dairy group D herds. Rotavirus was, after campylobacters, predominant in outbreak groups A, B and C and occupied third place as an agent in outbreaks of more than 4 calves but was significantly less commonly excreted by group D calves possibly due to effective vaccination. Coronavirus was detected in half of the outbreaks of more than 4 animals but was excreted by very few animals. On the other hand, K99<sup>+</sup>EC was present in only 4 of these herds but in 3 of them was isolated from more than 20 per cent of the calves examined showing that this agent is not very frequent in herds but is yet probably responsible for specific infections of very young calves; its absence in groups C and D was probably due to effective immunization of the dams.

Recovery of campylobacters is undoubtedly influenced by the period of storage of the faeces and the bacteriological procedures used. It was clearly shown how the isolation rate was significantly diminished in all outbreak groups when the faeces were stored at +4°C for 7 or more days (Table 4.20A). The overall isolation rate was likely to have been diminished by this effect and a further 31 calves would probably have been detected as campylobacter excretors from the negative 188 "old faeces". The expected theoretical overall isolation rate would have been 53.6 per cent (297/554).

More campylobacters were isolated from outbreak groups B and D calves than from A and C (second and first year procedures

respectively). Assuming that no natural epidemiological variations occurred in the field outbreaks during these 2 years studied the second year laboratory technique isolated 18 per cent more campylobacters. Nevertheless, significantly less "fresh faeces" were cultivated during the second than during the first year (Table 4.20C) and despite this the isolation rate of the second method was significantly higher than the first, showing that the improved methodology would have allowed the isolation of more than 18 per cent campylobacters. Applying a correction on the 371 faeces cultivated by the first year procedure we could deduce campylobacter excretion in 54.9 per cent of calves (377/687) and 67 extra animals would have been detected.

Such variable results in the isolation rates of ruminant campylobacters have been reported that there is no doubt that the different methodologies employed prevent comparison between many previous works and this survey. Firehammer and Myers (1981) by means of a filtration technique which favoured the isolation of jejuni/coli GC isolated campylobacters of this group, but no fetus GC, from 40.1 per cent of diarrhoeic calves. In this survey, using direct culture of faeces without filtration both groups of campylobacters were isolated from neonatal diarrhoeic outbreaks. The isolation rates were significantly affected by the bacteriological procedures used, as more jejuni/coli GC, many more fetus GC and more double Campylobacter spp. isolations were obtained by the second year procedure than the first (Table 4.19). During the second year of this survey, 35.5 per cent and 37.5 per cent jejuni/coli GC were isolated from groups B and D respectively (Table 4.12B)

which are similar to the 40.1 per cent obtained by Firehammer and Myers (1981). These figures are probably comparable because these authors also refrigerated the faeces for an unrecorded period of days before cultures were made. The work with the first procedure yielded fewer jejuni/coli GC and markedly less fetus GC. This phenomenon has a logical explanation as the plates were discarded as soon as the fast growing jejuni/coli GC appeared preventing the isolation of fetus GC which grow slowly on PN plates.

Due to the importance of the jejuni/coli GC in human enteritis advances in isolation techniques have been focused on this group of campylobacters and relatively little information has been published on the incidence of the fetus GC in the intestinal tract of ruminants. Interestingly, it has been demonstrated here that when appropriate bacteriological techniques are applied fetus GC can be isolated at similar levels to those of jejuni/coli GC.

Recently, work carried out at the same time as the author's was published (Morgan, Hall, Reynolds and Parsons, 1983) using Preston medium (Morgan, Personal Communication, 1983); 29.5 per cent of jejuni/coli GC were isolated from 827 normal and diarrhoeic calves. This percentage is nearly the same as the overall 29.3 per cent for jejuni/coli GC of this survey. They isolated 16.5 per cent of C. fetus subsp. fetus (fetus GC, HT) from their 827 calves which is also similar to the overall 18 per cent obtained here. In the present survey it was found that after C. jejuni in cattle, CHI was excreted by 14.8 per cent of the 687 calves examined but in outbreaks<sup>of</sup> group D was isolated from 29.1 per cent of the calves. Recently, Roberts, Allan, Walker

and Sommerville (1983) reported that the most common isolate in cattle are jejuni/coli GC, C. fetus subsp. fetus and CHI and although no isolation rates were published, it is interesting to note that this group of investigators also identified CHI strains in cattle. On the other hand, C. fetus was in this survey the least common isolate representing only 3.2 per cent of the overall isolations.

It was discovered in this study that dairy calves excrete significantly less campylobacters than beef calves (Table 4.14) whereas the isolation rates of the other enteropathogens in both groups was very similar. When the data was analysed for different Campylobacter spp. it was found that jejuni/coli GC were responsible for this difference (Table 4.15A). The isolation rate of the different fetus GC species separately considered was similar in both types of herds but a significantly lower number of calves excreted C. jejuni and to a lesser extent C. coli in dairy herds (Table 4.15B). Dairy herds represent 28.6 per cent of the calves investigated in outbreak A, 9.2 per cent of B, 10.8 per cent of C and 28.3 per cent of those of D. The first and second year methodologies did not influence these results as 52.5 per cent dairy calf faeces were cultivated according to the first year procedure and the remaining 47.5 per cent by the second year technique. These results were not influenced either by the period of storage of faeces (Table 4.20B) or antibiotic treatment (Table 4.15C). The exact age of each of the individual calves of this survey were not provided but in both types of herds similar age animals were sampled, always less than one month of life. So, the reason for the different excretion of campylobacter groups



between the two types of herd should be sought in the management and rearing of the animals. Dairy herd calves are separated from their dams and are fed with powdered or reconstituted milk.\* The lack of C. jejuni and C. coli excretion in dairy calves may indicate the key importance of the dam as source of early calf infection. The isolation rates of CHI and C. fetus in both types of herds shows that the epidemiology of these species should be different from jejuni/coli GC and possibly the dam is not so important for the transmission of infection. Perhaps the explanation of this different epidemiological behaviour of these two campylobacter groups is their different resistance in the environment; after excretion C. jejuni and C. coli may not survive so well as CHI and C. fetus and direct contact with recently excreted faeces could be required to initiate infection with the former group.

Overall comparisons of the excretion rates of jejuni/coli GC or fetus GC failed to show any significant difference between diarrhoeic and non-diarrhoeic animals (Table 4.18). Nevertheless, it should be emphasized that in this survey only 37 non-diarrhoeic calves from groups A and B were available for comparison and these controls, which had been taken from diseased herds, do not represent non-scouring animals from healthy herds. For this reason, comparisons will be made with animals from farms without enteric disorders at the time of sampling (vide infra).

When diarrhoeic and non-diarrhoeic excretion rates were compared it was found that rotavirus was the only enteropathogen in which a significant increase was detected in the affected animals, and this was found in outbreak groups A, B and C. *Cryptosporidium*

\* Such milk substitutes may be commonly expected to contain added substances active against some species of bacteria.



excretion rates were not significantly increased in diarrhoeic animals of groups A, B and C with respect to non-diarrhoeic A and B group calves.

In contrast with these two enteropathogens, campylobacter excretion rates were higher in non-diarrhoeic group A and B calves than in diarrhoeic animals although these differences were not highly significant ( $p < 0.05$ ). Prescott and Bruin-Mosch (1981) also isolated more jejuni/coli GC from healthy than from non-diarrhoeic calves. This phenomenon could be explained because campylobacter numbers are reduced in diarrhoeic faeces due to dilution by the fluid contents. This explanation lacks conviction and might only be plausible if the campylobacters were not the aetiological agent of the diarrhoea. It may also be that in some type of diarrhoeic disease calf faeces lose their natural mucus secretion, have changed pH or an increased content of lethal factors for campylobacters.

Group D outbreaks are quite different from the rest as calves excreted significantly less rotavirus and more cryptosporidia and campylobacters than any of the other groups (Table 4.12A). The absence of a significant number of calves excreting rotavirus indicates that the cause of diarrhoea in these animals from rotavirus vaccinated dams is not rotavirus unless rotavirus can induce diarrhoea in immunologically protected animals without the detection of virus excretion. Analysis of outbreaks of more than 4 animals also shows that in all of them cryptosporidium and campylobacters are the two infectious agents present in more than 20 per cent of the calves whereas rotavirus was only detected at this level in

one outbreak (Tables 4.21H and 4.21I). The question which arises is whether cryptosporidium or campylobacters alone are the cause of diarrhoea, both agents combined, or some as yet undisclosed agent. As no non-diarrhoeic group D calves were available for comparison the non-diarrhoeic animals from groups A and B have to be used for this purpose. Cryptosporidia were excreted by 50 per cent of 240 calves examined in group D (Table 4.12A) which is significantly more than the 13.5 per cent of the 37 non-diarrhoeic controls of groups A and B. On the other hand, if we compare the overall isolation campylobacter rate of group D, 145 out of 240 (60.4 per cent) calves (Table 4.12A) with the 19 out of 37 (51.3 per cent) non-diarrhoeic calves of groups A and B (Tables 4.17) we find that these differences are not significant (Yates' corrected  $\chi^2 = 0.748$ ,  $p < 0.50 > 0.30$ ). Furthermore, if to make the data more comparable, we correct the 31 group A controls for the second year methodology applying the aforementioned 18 per cent index and pooling the data, it is discovered that the corrected controls represent 22 out of 37 (59.4 per cent) calves positive for campylobacter which is nearly the same as the 60.4 per cent of group D. So, although campylobacters and cryptosporidium are predominant in group D calves, the most probable cause of diarrhoea are the cryptosporidium and not campylobacters.

Campylobacters were isolated more often from calves infected with Cryptosporidium sp. than from animals without detectable excretion of oocysts. In fact, 16.2 and 10.3 per cent more cryptosporidium infected calves respectively excreted fetus GC and jejuni/coli GC than animals without detectable parasites, these differences

being significant (Tables 4.24F and 4.24G). These results suggest that infection with Cryptosporidium sp. may favour the multiplication of campylobacters in the intestine.

Although in most group C calves rotavirus is probably a major cause of diarrhoea, this animal group occupies an intermediate position between groups A + B and D, probably due to some vaccinal immunity. In two outbreaks of 5 or more calves of this group, D520 and D530 (Table 4.21F), campylobacters were the only agent detected as significantly excreted by more than 20 per cent of the animals. Nevertheless, the campylobacter isolation rates of 40 and 50 per cent for these two herds are similar to the controls and so the cause of disease in these two farms remains undiagnosed.

Coronavirus was a widespread agent but was excreted by few calves. Only in herds D39, D360 and E421 was it excreted by 40, 22.2 and 55.5 per cent of the animals respectively (Tables 4.21A and 4.21H) which are figures significantly higher than in the non-diarrhoeic A and B controls (5.4 per cent, Table 4.17). The few calves with coronavirus infection from group D1 generally excreted significantly less campylobacters (Table 4.26) although the small number of animals prevents any conclusion.

K99<sup>+</sup>EC was excreted by 60, 42.9 and 25 per cent of the calves sampled in herds B2625, E97 and B2649 respectively, being in herds B2625 and E97 the only enteropathogen detected (Tables 4.21A and 4.21E). These figures are significantly increased as compared with the negative isolation rate of the 32 non-diarrhoeic groups A and B calves (Table 4.17). The absence of campylobacters in K99<sup>+</sup>EC positive calves (Table 4.25) probably merely indicates

that is a specific disease of very young calves.

In this survey it was demonstrated that diarrhoea and an increased detection rate were commonly associated for rotavirus and cryptosporidium, less frequent but nevertheless significantly in some specific outbreaks for K99<sup>+</sup>EC and coronavirus. On the other hand, it was not possible to correlate the presence of the most widespread agents, campylobacters, with the diarrhoeic disease as these bacteria were isolated both from scouring and the available non-scouring animals. Morgan, Hall, Reynolds and Parsons (1983) in a similar survey studied faeces of normal and affected calves from 44 outbreaks of neonatal diarrhoea and similarly to this survey they found that campylobacters were very commonly found and were associated with other bovine pathogens, but in no outbreak were campylobacters the only agent present. The overall isolation rate was similar, showing that the bacteriological methodology of both works was comparable, however they found that 20 and 34 per cent out of 463 diarrhoeic calves excreted C. fetus subsp. fetus (fetus GC, HT) and jejuni/coli GC respectively, whereas only 13 and 25 per cent of 364 non-diarrhoeic calves excreted these campylobacters. These differences were significant at  $p < 0.01$  by Chi-square test.

In Scotland, Roberts, Allan, Walker and Sommerville (1983) found that in cattle 58 per cent of typable jejuni/coli GC isolates belonged to one of the four Penner and Hennessy's serotypes: 4, 13, 16, 34, 43, 50; 2; 24 and 23. In this work, of the C. jejuni strains typed half belonged to the first group and the other half to the second common group with minor antigenic differences

and they were evenly distributed in the 3 farms studied.

C. jejuni was isolated from 23.6 per cent of the calves examined confirming the importance of cattle as a possible source of infection for humans. The serogroups 4, 13, 16, 50 and 2, which were found here, are responsible for human infections due to consumption of raw milk and were also isolated from cows in Colorado, USA (Blaser, Penner and Wells, 1982). Serotypes 1 and 2 are the most common among human isolates, 11.9 per cent of the isolates in British Columbia, Canada were serotype 2 and human isolates that react with both antisera 13 and 16 constitute 5 per cent of the total strains serotyped (McMyne, Penner, Mathias, Black and Hennessy, 1982).

From the 9 C. coli strains serotyped 7 had antigen 20 which had been recently described as specific for C. coli species (Penner, Hennessy and Congi, 1983) whereas the other two had some of the antigens described as specific for C. jejuni. Penner, Hennessy and Congi (1983) found that only 12 out of 282 (4.3 per cent) of C. coli isolates reacted with C. jejuni antisera, so the 2 out of 9 strains found in this work is considered a high proportion for hippurate negative strains. For these C. coli strains which are serologically related with C. jejuni Penner, Hennessy and Congi (1983) speculated that they are in fact C. jejuni which have lost their ability to hydrolyse hippurate.

### 3) Control farms survey

This survey was carried out in order to obtain information

on the campylobacter excretion rates of non-diarrhoeic young calves from herds without enteric disorders at the time of sampling. Part of this work has been reported previously as a comparison between PN and RNBGT media (Chapter 3, Section C).

#### Materials and Methods

Fifty nine calves were sampled from 3 dairy farms identified as A, B and C. In farm A calves had ages varying between 7 to 37 days; in farm B between 40 and 45 days and in farm C between 25 and 43 days. No animal was diarrhoeic at the time of sampling. No enteric disease had been diagnosed in farms A and C but in farm B a few animals had had diarrhoea at 7 to 10 days of life, in all cases 30 days before the samples were taken.

Faeces were collected from the rectum as described in Chapter 2 and were plated on PN plates on the same day of collection at the farms. All samples were processed according to the second year laboratory methodology. In order to make the results comparable to the A.D.R.A. survey the extra campylobacters which were isolated by means of RNBGT in that work but not obtained from the corresponding PN plates of the same animal will be disregarded (in Table 3.8 only first column considered).

#### Results

In farm B all animals excreted campylobacters, in farm C 86.7 per cent and in farm A 15.4 per cent (Table 4.27). Jejuni/coli GC was isolated from 66.7, 13.3 and 3.8 per cent of the calves of farms B, C and A respectively. Fetus GC was isolated from 86.6, 61.1 and 11.5 per cent of the calves of farms C, B and A respectively. All campylobacter species found in A.D.R.A. survey

were isolated, 2 strains could not be typed due to contamination. Overall 35 out of 59 (59.3 per cent) calves excreted campylobacters and in 7 animals two species were detected. Seventy one per cent and nearly 43 per cent of calves excreted the fetus and jejuni/coli GC respectively.

### Discussion

All faeces were cultivated on the day of collection so in this case the high isolation rate therefore represents a true excretion rate. The second year methodology was also applied which is a more accurate evaluation of campylobacter excretion.

In farm B all calves excreted campylobacters despite the absence of diarrhoea. However, twelve of these animals had suffered diarrhoea one month before the sampling soon after their arrival at the farm at 3 days of life, the other six had not suffered from clinical enteric disease.

The different isolation rates on the 3 farms indicates that the occurrence of campylobacter excretion varies with time or between farms. In farm A fourteen calves were 7 days of life or less, thirteen 10-11 days and seven were between 21 and 37 days. The lower isolation rate in farm A calves could be due, in part, to a higher proportion of younger animals.

Difference exists in the relative proportion of fetus GC and jejuni/coli GC between these farms (Table 4.27). For instance, farms A and C have a higher isolation rate of fetus GC than jejuni/coli GC whereas the proportion of both campylobacter groups in farm B was similar. Farm B differs from the other control farms and also from the general characteristic found in A.D.R.A. survey

that dairy farms have less proportion of jejuni/coli GC, but it has to be noted that calves from this farm were older than those of the other farms considered.

### General Discussion

Despite the difference in age the diarrhoeic animals of the diagnostic survey and the diarrhoeic calves of the A.D.R.A. survey had very similar campylobacter isolation rates. In the diagnostic survey 45.9 per cent (39/85) and in the A.D.R.A. survey 45.6 per cent (277/608) animals excreted campylobacters. If the non-diarrhoeic calf isolation rates are compared the control farms are quite similar (59.3 per cent, 35/59) to the non-diarrhoeic calves of the A.D.R.A. (51.4 per cent, 19/37) survey but identical when the latter are corrected for the methodology employed, 59.4 per cent (22/37). The diseased non-diarrhoeic animals of the diagnostic survey have a lower isolation rate (27.6 per cent) than the other group of non-diarrhoeic animals but not significantly so on account of the small number of animals available for comparison.

When diarrhoeic and non-diarrhoeic animals of both surveys were pooled it was found that 45.6 per cent of 693 diarrhoeic animals excreted campylobacters and in 49.6 per cent of 125 non-diarrhoeic animals the bacteria were detected (Table 4.28A). Correcting the data of the campylobacter negative animals for the second year methodology (+18 per cent) it was found that 52.7 and 55.2 per cent would have been the isolation rates of diarrhoeic



and non-diarrhoeic animals respectively (Table 4.28B). It is concluded therefore that there is no difference between the overall campylobacter isolation rates of diarrhoeic and non-diarrhoeic cattle.

When different campylobacter groups were compared for pooled data of the 3 bovine surveys it was found that 29.7 per cent (206/693) diarrhoeic and 24.8 per cent (31/125) non-diarrhoeic animals excreted jejuni/coli GC; 18.3 per cent (127/693) diarrhoeic and 26.4 per cent (33/125) non-diarrhoeic animals excreted fetus GC. The differences were not significant by Yates' corrected Chi-square test for jejuni/coli GC ( $\chi^2 = 1.021$ ,  $p < 0.50 > 0.30$ ) but with low significance for the higher isolation rate of fetus GC from the non-diarrhoeic pooled animals ( $\chi^2 = 3.889$ ,  $p < 0.05 > 0.10$ ). Nevertheless, the difference is due to a higher isolation rate of the fetus GC from the control farms groups 42.4 per cent (25/59) whereas from the rest of the non-diarrhoeic animals of the other two surveys only 12.1 per cent (8/66) excreted fetus GC. As the faeces of the control farms were processed by the second year methodology and were the only ones cultivated a few hours after sampling this difference could be merely due to technical causes.

Overall, 28.7 per cent (247/860) of the animals were shedding jejuni/coli GC confirming the possible importance of cattle as reservoir of infection for humans.

CHI isolates were very common as 15.6 per cent (134/860) cattle excreted this microorganism whereas C. fetus was detected in only 3.4 per cent (29/860) of these animals. It has to be pointed out

that H<sub>2</sub>S producing CHI strains have never been isolated from abortions whereas C. fetus subsp. fetus (C. fetus) is found in sporadic bovine abortions, so it may be important to differentiate between these two types as their pathogenicity could be distinct. If this is true the real abortigenic C. fetus strains may in fact not be so common in the intestinal tract of cattle as was previously believed. For instance, El Azhary (1968) isolated "Vibrio fetus intestinalis" from 22.4 per cent non-diarrhoeic bovine faeces which is very similar to 26.4 per cent non-diarrhoeic cattle in which fetus GC were detected here. Many of the El Azhary's strains may have been CHI and this could explain why only a few of his "V. fetus intestinalis" were able to induce placentitis and abortion and most of the strains isolated from bovine faeces in herds with normal fertility or from swine faeces did not multiply in the genital tract. Similarly, the strains recently described by Morgan, Hall, Reynolds and Parsons (1983) as C. fetus subsp. fetus probably includes many CHI.

Interestingly, "C. fecalis" and another similar bacterium, C. sputorum subsp. bubulus, were not isolated from any of the cattle examined in the three surveys. Although the technique here was sub-optimal for the isolation of these two species, the same bacteriological techniques isolated "C. fecalis" in the ovine surveys. These bacteria were not even isolated when a more suitable medium, RNBGT, was used to attempt their recovery in 152 calves of the A.D.R.A. and control farms surveys (Chapter 3, Section C). Nevertheless, C. sputorum subsp. bubulus have been isolated from bovine faeces (Florent, 1959b; Lederle, 1963; Clark,

Monsbourgh and Dufty, 1969; Mehle, 1971; Hasselbach, 1982) so perhaps this bacterium was present in very low numbers and remained undetected. On the other hand, there is confusion because in recent publications the strains previously isolated from cattle and provisionally classified as "C. fecalis" (Al-Mashat and Taylor, 1980a) are in fact CHI, a subject which will be discussed in detail in Chapter 5.

CHAPTER 5TAXONOMY AND BIOTYPING

As stated earlier there has been controversy and confusion in the nomenclature of the genus Campylobacter. The essential features attributed to the genus were that it should comprise Gram-negative slender, curved bacteria, motile with a single polar flagellum, microaerophilic with a strictly respiratory metabolism, acid not produced in media with carbohydrates and DNA base composition between 29 and 36 per cent (Véron and Chatelain, 1973). Ten years later due to the discovery of new campylobacters and recent developments in the classification and identification of species some of the characters of this officially adopted definition (Skerman, McGowan and Sneath, 1980) are now considered obsolete. For instance, C. fetus subsp. venerealis and "C. pyloridis" contain lophotrichate cells - up to five flagella at one pole (Skirrow, Purdham and Benjamin, 1983; Marshall and Warren, 1983), other campylobacters are able to grow aerobically (Neill, O'Brien and Ellis, 1983) and recently it has been suggested that the range of the DNA base composition of the genus be extended to between 29 and 41 mol per cent G+C (Owen, 1983).

The campylobacters studied here are microaerophilic and represent a group of typical campylobacters as defined by Véron and Chatelain (1973). Advances have been made in the taxonomy mainly due to the growing importance of human campylobacter enteritis but this research has been largely restricted to the jejuni/coli GC whereas other species of microaerophilic campylobacters have received little attention. Amongst these other campylobacters is a group

which will be provisionally called in this thesis fetus GC, a group name to identify microaerophilic campylobacters different from C. jejuni, C. coli or C. laridis which have been isolated from cattle and sheep.

Changes in methodology have been adopted throughout this research in order to develop a typing scheme to classify both jejuni/coli GC and fetus GC and also to improve the isolation rates by detecting mixtures of campylobacters. These changes included modifications of some selected tests and the addition of others. Two biotyping schemes have been used during the first and second year respectively to identify C. jejuni, C. coli and fetus GC. Whenever possible all isolated campylobacters were stored frozen and this procedure has allowed the author to reclassify all fetus GC strains or to repeat tests where necessary.

The purposes of this study were to classify the campylobacters strains isolated from the bovine and ovine surveys, develop a simple typing scheme to identify species of both jejuni/coli GC and fetus GC and compare the results of the bovine and ovine isolates with a limited number of selected standard and non-standard strains from other origins.

## CHAPTER 5: SECTION A

### GENERAL BIOTYPING SCHEMES

In this section, the tests used to classify all the strains will be detailed. Other tests and particular studies which were carried out on a limited number of strains will be separately treated in the next section.

Before describing the biotyping schemes themselves it is necessary to consider some aspects of the tests. An explanation of the use of each test and the reason why it was chosen along with modifications is given. The aim was to use as few useful tests as possible because it was important to biotype a substantial number of strains.

Since this work was started in September 1981 the original taxonomic criteria have been modified. This was due to new information on DNA relatedness which has altered the species significance of some tests and as a result of the experience of the first year of biotyping. At the start of this work biotyping was based on the work of Skirrow and Benjamin (1980a and b), Karmali, de Grandis and Fleming (1980) and Karmali, Allen and Fleming (1981). The first named authors' tests for hippurate hydrolysis,  $H_2S$ -FEP production, tolerance to TTC and NaI and growth at 25°C and 43°C were employed along with Karmali's tests for C sensitivity, microscopic morphology, swarming and coccal transformation.

#### 1) Hippurate hydrolysis

C. jejuni is unique in being able to hydrolyse hippurate. The test is especially useful as few other tests differentiate

C. jejuni from C. coli which has similar colonial morphology, microscopic morphology and temperature tolerance. C. jejuni and C. coli had been classified as distinct species by Véron and Chatelain (1973) and this separation officially approved (Skerman, McGowan and Sneath, 1980) however when this work was undertaken it was still doubtful whether these two campylobacters were different species or merely biotypes. The hippurate test was adapted to campylobacters by Harvey (1980) and later modified by Skirrow and Benjamin (1981a). In 1981 Véron proposed that the hippurate characterisation of jejuni/coli GC strains should be provisionally suspended until DNA hybridization studies were completed (Newell, 1982). To-day these studies have demonstrated that C. jejuni and C. coli are separate species and the hippurate test highly correlates with the DNA relatedness (Harvey and Greenwood, 1983; Owen, 1983).

## 2) Hydrogen sulphide production

2.1 Iron medium: During the first year of work this test was used as recommended by Skirrow and Benjamin (1980a and 1982) for subdividing C. jejuni into biotypes 1 ( $H_2S$  producer) and 2 ( $H_2S$  non-producer). This test was abandoned in the second year for C. jejuni when it became known that the ability of C. jejuni to produce  $H_2S$  from FBP compound is not correlated with any significant difference between the DNA contents of the two biotypes (Leaper and Owen, 1982). Nevertheless, this test was found to be useful for a different purpose and during the second year it was used to differentiate "C. fecalis" from C. fetus and CHI.

This test was modified from that of Skirrow and Benjamin (1982),

by the final reading being made after overnight incubation at  $32^{\circ}\text{C}$   $\rightarrow$   $\text{O}_2$  and minimal exposure of the campylobacter plate growth to air as indicated in Chapter 2. These modifications were made after preliminary tests had shown that fluctuation of temperature could affect the results and that plates of "positive" C. jejuni strains exposed to air for more than 1 hour yielded weakly positive or eventually negative results.

2.2 Triple Sugar Iron (TSI): The production of  $\text{H}_2\text{S}$  in media such as TSI, Kligler or Sulphide-indol-motility medium (SIM) has been used as an "insensitive" test to differentiate species. Both C. fetus subsp., C. jejuni and C. coli fail to produce  $\text{H}_2\text{S}$  whereas the three C. sputorum subsp. and "C. fecalis" produce abundant  $\text{H}_2\text{S}$  in these media (King, 1957; Smibert, 1965b and 1974; Firehammer, 1965 and 1979; Berg, Jutila and Firehammer, 1971; Lawson, Rowland and Roberts, 1976; Hasselbach, 1982). Nevertheless, strains isolated from pigs formerly described as C. coli type I (Lawson, Rowland and Wooding, 1975) and later provisionally named CHI by Gebhart, Ward, Chang and Kurtz (1983) closely resemble C. fetus subsp. fetus but are able to produce  $\text{H}_2\text{S}$  from TSI. In this work TSI medium was introduced to re-assess the fetus GC in order to distinguish between C. fetus, CHI and "C. fecalis".

Screw-caps of TSI McCartney bottles were removed and replaced by cotton plugs so that the incubation was at  $37^{\circ}\text{C}$ - $650\text{H}_2$  for 2 days in a hydrogen rich atmosphere which enhances  $\text{H}_2\text{S}$  production (Jorgensen, 1983). Care was taken to use fresh, recently prepared media, with no more than 7 days of storage, as recommended by



Gebhart, Ward, Chang and Kurtz (1983).

### 3) Tests of chemical and antibiotic tolerance

They were performed by the Skirrow and Benjamin (1980b) technique as described in Chapter 2.

3.1 2,3,5-triphenyl-tetrazolium chloride (TTC): Véron and Chatelain (1973) stated that 9 strains of C. coli were able to grow on blood agar containing 0.1 per cent (w/v) of TTC whereas C. jejuni strains grew irregularly on this medium (Florent, Personal Communication to Véron and Chatelain, 1973). Later, Skirrow and Benjamin (1980b) improved this test by the use of impregnated strips of blotting paper with 4 per cent (w/v) of TTC as has been described in Chapter 2. They classified strains giving inhibition zones of 6mm or more as sensitive and less than 6 mm as resistant. The same criterion was adopted in this work.

TTC sensitivity test was used as a complementary test to differentiate jejuni/coli GC from fetus GC and C. jejuni from C. coli. It was also employed to investigate the sensitivity of C. fetus, CHI and "C. fecalis" strains. Later, this test was also found useful in the separation of mixtures of C. jejuni and C. coli.

Kuhn and Jerchel (1941) found that tetrazolium compounds are reduced to deeply coloured formazan derivatives by growing bacteria. Later, Usdin, Shockman and Toennies (1954) used tetrazolium salts to increase the visibility of zones of bacterial growth and for that purpose pointed out that TTC is reduced to red formazan derivatives which are superior to other tetrazolium salts. They also demonstrated that oxygen prevents the formation of red formazan

by bacteria. In this test the TTC diffuses from the strip into the agar and the growth of campylobacters in an atmosphere of 3 per cent oxygen is clearly seen as a red band. As TTC is decomposed by visible or ultraviolet light (Dawson, Elliott, Elliott and Jones, 1969) care was taken to protect the strips from light exposure.

3.2 Nalidixic acid (Nal): Véron and Chatelain (1973) demonstrated that C. coli and C. jejuni were not able to grow on blood agar containing 40 µg of Nal per ml whereas C. fetus subsp. consistently grew on this medium. Later, this observation was confirmed and 30 µg Nal discs were used as a reliable routine test to differentiate C. jejuni and C. coli from C. fetus subsp. (Skirrow and Benjamin, 1980b; Karmali, de Grandis and Fleming, 1980; Hébert, Hollis, Weaver, Lambert, Blaser and Moss, 1982).

3.3 Cephalothin (C): Cooper and Slee (1971) discovered that a campylobacter strain isolated from the blood of a patient with diarrhoea was resistant to cephalothin and subsequently used C discs to eliminate contaminants and isolate campylobacters by direct culture of faeces onto blood agar plates. Karmali, de Grandis and Fleming (1980) after an antimicrobial sensitivity study in which the minimum inhibitory concentration of 60 jejuni/coli GC and 12 C. fetus subsp. fetus strains were obtained for seven cephalosporins and cefoxitin, proposed the use of 30 µg C and Nal discs for routine identification of these campylobacters. In their study 59 out of 60 jejuni/coli GC strains produced no zone of inhibition whereas in contrast the 12 C. fetus subsp. fetus strains were inhibited around the 30 µg C discs.

#### 4) Temperature tolerance tests

When McFadyean and Stockman (1909) described the first bacteria of the genus Campylobacter they observed that it grew best between 30° to 37°C but also slowly at room temperature. Later, Kuzdas and Morse (1956) discovered that a bovine Campylobacter spp. strain isolated from an outbreak of bovine dysentery was able to grow at 45°C. King (1957 and 1962) suggested the use of temperature of incubation to distinguish between human jejuni/coli GC and C. fetus subsp. fetus strains; the former strains grew luxuriantly at 42°C but failed to grow at 25°C whereas the latter grew at 25°C but showed little or no growth at 42°C. When King's test was applied to bovine and ovine C. fetus subspp. strains great variability was encountered (Firehammer and Berg, 1965) whereas ovine jejuni/coli GC strains behaved typically (Smibert, 1965). El Azhary (1968) did not obtain any difference of growth either at 37°C, 42°C or 45°C between jejuni/coli GC and fetus GC strains isolated from bovine, ovine, porcine or avian faeces. Nevertheless, Al-Mashat and Taylor (1980a) found that a few bovine jejuni/coli GC or C. fetus subsp. fetus strains behaved as described by King.

King (1957) also described that C. sputorum subsp. bubulus strains grew at 25°C and 37°C but only very lightly at 42°C. Afterwards, Hasselbach (1982) observed that bovine faecal C. sputorum strains were able to grow at 25°C and 43°C. Firehammer (1965) showed that ovine "C. fecalis" strains grew at 37°C and 42°C but only poorly or not at all at 25°C.

Gebhart, Ward, Chang and Kurtz (1983) described porcine CHI

strains which grew well at 25°C but poorly at 43°C whereas in this work the porcine CHI NCTC11562 grew luxuriantly at 43°C (vide supra Chapter 3, Section A). Al-Mashat and Taylor (1980b) isolated a CHI strain (named "C. fecalis") which was able to grow at 42°C and 43°C but not at 25°C and another similar strain which was able to grow either at 25°C, 42°C or 43°C and for that reason was called "C. unidentified".

Véron and Chatelain (1973) used King's test to differentiate jejuni/coli GC from C. fetus strains in the scheme later approved and officially recognized (Skerman, McGowan and Sneath, 1980). To-day laboratories (Skirrow and Benjamin, 1980b; Hébert, Hollis, Weaver, Lambert, Blaser and Moss, 1982) have confirmed the value of King's test and found it useful to separate jejuni/coli GC from fetus GC. Skirrow and Benjamin (1980b) pointed out that the 25°C test gave 100 per cent separation between these two campylobacter groups, whereas less consistent results were found at the higher temperature which they recommended be performed at 43°C instead of 42°C in order to increase the accuracy.

Taking into account laboratory variation the divergent results reported might indicate a difference in temperature tolerance between bovine and ovine fetus GC with respect to human C. fetus subsp. fetus. This study was undertaken to examine the temperature tolerance of C. fetus, CHI and "C. fecalis" and for biotyping ruminant campylobacters only during the first year of work. The test was performed in a water bath placed in a cold room maintaining a constant maximum temperature of 25°C (Chapter 2).

### 5) Coccal transformation

McFadyean and Stockman (1909) described the morphology of the first reported campylobacter, "in older cultures the vibrio presents a granular appearance, and many of the elements have broken up into small granules of a circular shape which usually take the stain feebly". Later, Smith and Taylor (1919) with C. fetus, Firehammer and Lovelace (1961) and Firehammer (1965) with C. fetus subsp. fetus, C. sputorum subsp. bubulus and "C. fecalis" found that some old cultures developed coccal forms which frequently possessed a flagellum. Gebhart, Ward, Chang and Kurtz (1983) observed that overincubated CHI cultures produced coccoid forms.

Jones, Orcutt and Little (1931) noted that when jejuni/coli GC cultures became older, clumps occurred and granule formation was the rule and Florent (1959b) found that jejuni/coli GC strains degenerated more easily than other campylobacter groups.

Recently investigators have realized that the ability of Campylobacter spp. to produce coccal bodies can be used for taxonomic purposes. Skirrow and Benjamin (1980b) used the natural formation of coccal forms to distinguish between C. fetus subsp. fetus (scarce), jejuni/coli GC (common) and C. lariidis (very common). Karmali, Allen and Fleming (1981) discovered that the cause of granular degeneration was the exposure of the bacterium to oxygen and confirmed that Campylobacter spp. formed coccal bodies at different rates. They standardized a simple test in which petri dishes cultures were exposed to air for 6 days and named it coccal transformation (Chapter 2). In this test jejuni/coli GC consistently showed rapid coccal transformation whereas C. fetus

subsp. venerealis and C. fetus subsp. fetus displayed a predominantly normal morphology with very few or no coccal forms.

Coccal transformation was carried out during the first year of this work as an additional test to differentiate jejuni/coli GC from fetus GC (Figures 5.7, A and B). A delayed coccal transformation test was also used with a limited number of strains to assess whether differences in coccal transformation could be detected between C. fetus, CHI, "C. fecalis" and C. sputorum subsp. bubulus.

#### 6) Catalase test

The genus Campylobacter is broadly divided into two main groups the catalase positive and negative campylobacters (Véron and Chatelain, 1973). Jejuni/coli GC, C. fetus subsp. fetus and CHI are catalase positive campylobacters the first two of which have been frequently isolated from the intestinal tract of cattle and sheep (Florent, 1963; Smibert, 1965b; El Azhary, 1968; Clark, Monsbrough and Dufty, 1969; Allsup and Hunter, 1973; Al-Mashat and Taylor, 1980a; Firehammer and Myers, 1981; Prescott and Bruin-Mosch, 1981). "C. fecalis" is a catalase positive campylobacter similar to C. sputorum subsp. bubulus which have been isolated from the intestinal tract of sheep (Firehammer, 1965 and 1979).

C. sputorum subsp. bubulus is a catalase negative campylobacter which has occasionally been encountered in the intestinal tract of healthy cattle (Florent, 1959b; Lederle, 1963; Mehle, 1971; Clark, Monsbrough and Dufty, 1969; Hasselbach, 1982). Vandenberghe and Hoorens (1980) isolated a catalase negative unclassified campylobacter from sheep suffering from proliferative enteritis.

In general, catalase production correlates well with DNA relatedness data (Owen, 1983) but this characteristic is not infallible as strains of C. sputorum subsp. bubulus and "C. fecalis" differed only in this test but were genotypically and otherwise phenotypically indistinguishable (Harvey and Greenwood, 1983).

This test was included during the first year of work to classify all bovine and ovine isolates, later, it was specifically used to compare the catalase activity of "C. fecalis" and C. sputorum subsp. bubulus isolated from the intestinal tract of sheep and the genital tract of cattle.

#### 7) Swarming and consistency of growth

Campylobacters are generally motile bacteria and many of these microorganisms have a tendency to spread and form coalescent colonies especially on moist agar or media with low agar concentration. Differences in motility have allowed campylobacter groups to be identified on the basis of their swarming (Karmali, Allen and Fleming, 1981). Jejuni/coli GC and C. laridis are highly motile campylobacters which spread readily whereas fetus GC are less motile bacteria. C. fetus subsp. fetus and porcine CHI are less effuse than jejuni/coli GC and lack the metallic sheen commonly found in jejuni/coli GC (Gebhart, Ward, Chang and Kurtz, 1983). "C. fecalis" and C. sputorum subsp. bubulus form individual non-spreading colonies (Florent, 1959b; Firehammer, 1965).

#### 8) Microscopic morphology

Florent (1959b) observed differences in the microscopic characteristics of bovine C. fetus and C. sputorum subsp. bubulus and this work was later supported by Firehammer and Lovelace (1961)



who studied ovine strains. Firehammer (1965) and Smibert (1974) found that "C. fecalis" isolated from the intestinal tract of sheep was microscopically very much like C. sputorum subsp. bubulus being predominantly short comma shaped cells with rounded ends which did not show the sharp curved forms of C. fetus.

King (1962) compared the morphology of C. fetus and jejuni/coli GC cells. She found that C. fetus were long with few undulations whilst jejuni/coli GC were short curved rods with short wavelength in spiral form. Later on, Karmali, Allen and Fleming (1981) measured the wavelength and amplitude of jejuni/coli GC, C. fetus subsp. fetus and C. fetus subsp. venerealis confirming the previously described morphological differences between jejuni/coli GC and C. fetus and discovering notable differences between C. fetus subspecies. They found that the differences in wavelength between these groups were highly significant jejuni/coli GC having the smallest wavelength and amplitude and C. fetus subsp. venerealis the biggest. Furthermore, they were able to distinguish between strains of these 3 groups by the use of phase-contrast microscopy without actually measuring the size.

In this thesis the microscopic appearance of carbol-fuchsin smears was used as an initial step in the identification of campylobacter isolates from CBA cultures and at the same time to differentiate between 3 groups jejuni/coli GC (C. jejuni and C. coli), fetus GC (C. fetus and CHI) and "atypical" fetus GC (C. sputorum subsp. bubulus and "C. fecalis").



### Biotyping Schemes

During the first year of this research a biotyping scheme was developed combining selected tests of the Skirrow and Benjamin (1980a and b); Karmali, de Grandis and Fleming (1980) and Karmali, Allen and Fleming (1981) procedures. The isolated strains were first cloned and stored frozen and then tests were carried out in batches of 30 or 40 strains at a time. This scheme was found unsuitable for the classification of campylobacters different from C. jejuni or C. coli and it had become clear that the sub-division of C. jejuni into biotypes would not be important either genotypically (Leaper and Owen, 1982) or epidemiologically as C. jejuni biotype 2 was rarely encountered in the cattle and sheep examined. These findings prompted the development of a simplified scheme or screening test which allowed the quick and reliable identification of C. jejuni and C. coli using only a few tests. Other campylobacters (fetus GC) were stored frozen for further identification at a later date. The aim was to quickly classify strains and for that reason uncloned cultures directly sub-cultured from the selective plates were used for the screening test which was carried out weekly.

When the surveys were finished work was focused on the biotyping of the frozen fetus GC strains. First, a study was carried out with representative isolates which were compared with standard strains in some selected tests. As a conclusion of this work, a few tests were chosen to reclassify all the remaining frozen fetus GC strains in a quick newly developed fetus screening test. Later, more detailed comparative studies were carried out using

a few representative fetus GC isolates and standard and other strains of this group, in order to evaluate tests of taxonomic value.

The campylobacter strains biotyped here were the strains isolated either from P, PN, S, SN or RNBGT during the ovine and bovine surveys previously described in Chapter 4. Overall 523 strains from the surveys were biotyped of which 306 were jejuni/coli GC and 217 fetus GC (Table 5.1). Details of the survey origin and final classification of these strains are shown in Tables 5.2 and 5.3.

#### 1) Biotyping scheme

The purpose of this scheme was to identify C. jejuni biotype 1 and 2, C. coli, C. laridis, C. fetus and some atypical C. fetus strains as suggested by Skirrow and Benjamin (1980a and b). This scheme was used in combination with the first year procedures (Chapter 4).

#### Materials and Methods

Nal and TTC tolerance tests, catalase, growth at 25°C and 43°C, spread, consistency and features of growth, coccal transformation and microscopic morphology were carried out on 222 campylobacter strains from the bovine and ovine surveys. Only 166 campylobacter strains were tested for C sensitivity as follows: 94 C. jejuni, 33 C. coli, 13 CHI type 1, 5 CHI type 2, 6 C. fetus and 15 "Atypical" fetus GC. Hippurate hydrolysis and H<sub>2</sub>S-FBP production tests were carried out from growth of only 160 previously identified jejuni/coli GC strains on CBA incubated at 43°C-550H<sub>2</sub> for 18 hours. All other tests were performed from growth obtained from CBA plates

incubated at  $37^{\circ}\text{C}$ - $650\text{H}_2$  for 48 hours. Hippurate hydrolysis tests were read after only 2 hours incubation and all doubtful results were repeated. All tests were carried out as described in Chapter 2.

*Campylobacter* standard strains used as controls are shown in Table 2.2.

### Results

Jejuni/coli GC was an homogeneous group as all 160 isolates consistently were positive in catalase (Figure 5.8) and coccal transformation tests (Figures 5.7A and B), were sensitive to Nal but resistant to C (Figures 5.5 and 5.6), grew at  $43^{\circ}\text{C}$  but not at  $25^{\circ}\text{C}$ , had a characteristic metallic sheen of growth and all but 8 strains spread readily (Table 5.4). On the other hand, fetus GC was more heterogeneous and two groups were clearly distinguished. One, the "typical" fetus GC was composed of 47 strains of bovine origin which were all catalase positive, negative coccal transformation test, were sensitive to C but resistant to Nal, displayed a variable growth at  $43^{\circ}\text{C}$  or  $25^{\circ}\text{C}$ , growth had a superficial matt aspect without metallic sheen and although spread occurred in some strains it was recognizably weaker than that of jejuni/coli GC. The other group was provisionally denominated "atypical" fetus GC and was composed of 15 strains of ovine origin which were distinct from the rest: the catalase and coccal transformation tests were variable, they were either sensitive to both Nal and C or did not grow on agar for tolerance tests, grew at  $43^{\circ}\text{C}$  but not at  $25^{\circ}\text{C}$ , did not swarm and had a unique sticky and adherent growth on CBA plates (Table 5.4).

Microscopic observation of carbol-fuchsin smears was complementary: fetus GC strains had the biggest and longest cells with long undulations, "atypical" fetus GC were short but coarse and wide with few long undulations that usually appeared straighter than fetus GC and jejuni/coli GC were the smallest campylobacters, short, thin with a typical recognizable short wavelength (Table 5.4).

C. jejuni was separated from C. coli by means of hippurate (Figure 5.1) and TTC tolerance (Figure 5.4), there was substantial agreement between these tests. C. jejuni was subdivided into biotypes by detection of  $H_2S$ -FBP production (Figure 5.2, Table 5.5). Overall of the 114 hippurate positive C. jejuni only 6 (2.7 per cent) were found to be  $H_2S$ -FBP positive C. jejuni biotype 2, the remaining 108 strains were biotype 1. Four C. jejuni strains were considered to be hippurate positive but reacted weakly (Figure 5.1). Forty six hippurate and  $H_2S$ -FBP negative C. coli strains (20.7 per cent) were identified. Twenty eight strains were completely resistant to TTC ( $R = 0$  mm) and 15 strains had zones of 6 mm or less which were classified therefore as resistant according to the criterion adopted; nevertheless, 3 C. coli strains had TTC sensitivity zones of more than 6 mm and they were considered sensitive.

The temperature tolerance test was not found to be suitable for subdividing the fetus GC (Table 5.6). Only 21 strains grew typically at  $25^{\circ}C$  but not at all or very weakly at  $43^{\circ}C$ , further work identified these strains as 12 CHI and 9 typical C. fetus (vide infra). Of the remaining strains, 21 grew either at  $43^{\circ}C$

and 25°C, 18 grew only at 43°C and 2 failed to grow at either temperature and only grew when the plates were incubated at 37°C. The "atypical" fetus GC strains which grew at 43°C but not at 25°C only did so when the test was carefully performed as described in Chapter 2. Nevertheless, 3 strains grew weakly at 25°C.

All but 3 fetus GC strains were sensitive to C and all without exception were resistant to Nal. All but 4 fetus GC 2 (CHI) were sensitive to TTC (more than 6 mm of inhibition haloes) (Table 5.6). Details of the measurements of the inhibition haloes will be referred to later on, together with the results of the screening test.

All campylobacters but 3 strains of the "atypical" fetus GC were catalase positive. These 3 strains belonged to the provisionally denominated atypical 1 and 2 strains which were later classified as C. sputorum subsp. bubulus (Table 5.6). One "C. fecalis" strain was catalase positive but reacted very weakly, the other "C. fecalis" were strongly catalase positive. Five C. jejuni biotype 1 reacted producing a weakly positive catalase test, all the other campylobacters were frankly positive.

Jejuni/coli GC and fetus GC were detected together in 4 animals of the A.D.R.A. and diagnostic surveys (Chapter 4). Two double isolations of C. jejuni biotype 1 and CHI type 1 and one of C. jejuni biotype 1 and CHI type 2 were directly detected on PN plates. The other was accidentally discovered when despite cloning it transpired that was a mixture of two campylobacters, C. jejuni biotype 2 and CHI type 1, which initially was considered to be an unclassified strain.

## Discussion

This work led to the identification of three major groups of campylobacters. Although this scheme was useful in the classification of the jejuni/coli GC species the taxonomy of the fetus GC was still confused. In fact, more than the necessary tests for identification of C. jejuni and C. coli were carried out in this scheme <sup>; strains</sup> could quickly and accurately be classified by means of TTC, Nal, C, hippurate, microscopic morphology, swarming, metallic sheen and consistency of growth. Besides, these same few tests and characteristics would allow differentiation of the fetus GC or the atypical fetus GC in the same way as the complete scheme.

## 2) Screening Scheme

In order to simplify the procedure future isolates were not initially cloned and were classified by a screening test which will next be described. This scheme was used in combination with the second year procedure already described (Chapter 4) to simply separate C. jejuni, C. coli and fetus GC.

## Materials and Methods

Nal, TTC and C tolerance tests and hippurate hydrolysis tests were carried out on growth from single colonies without further cloning, campylobacters were typed at this stage or from frozen stock. All cultures were grown on duplicate CBA plates at 37°C-650H<sub>2</sub> for 2 days. Other characteristics such as microscopic morphology, swarming, metallic sheen and growth consistency were

also recorded and they were used as complementary characteristics for identification. These tests were carried out in 301 strains isolated from PN or RNBGT media from the A.D.R.A. and control farms surveys (Table 5.1). From 3 A.D.R.A. calves 9 extra C. jejuni colonies were tested by this screening test as part of a work of re-isolation of C. jejuni from C. coli mixtures. Another sixteen C. jejuni cloned strains were included in this screening test; they were re-isolated from gnotobiotic calves and lambs for a study on motile (type M) and non-motile (type N) C. jejuni strains (Chapter 6).

In 296 strains hippurate test was read after 10 and 120 minutes of incubation at room temperature and in the other 30 campylobacters it was read only at 2 hours. In both cases all doubtful results were repeated.

Campylobacter standard strains were used as controls as shown in Table 2.2. All tests were carried out as described in Chapter 2.

#### - Technique for detection of campylobacter mixtures

Jejuni/coli GC + fetus GC mixtures: when a strain appeared resistant to both C and Nal an attempt was made to detect the presence of more than one species. C. jejuni or C. coli were re-isolated from the growth adjacent to the C disc and fetus GC were similarly obtained from the Nal disc. Presumptive diagnosis of the mixture was made by observation of Gram smears from growth adjacent to Nal discs and located at more than 25 mm from the Nal disc. In mixtures, growth adjacent to Nal had typical fetus GC morphology whereas the other was a mixture of predominantly coccal forms



and occasional fetus GC campylobacters.

C. jejuni + C. coli mixtures: these mixtures were detected as double layers of growth on TTC tolerance plates (Figure 5.9). Pure hippurate negative C. coli could be re-isolated from the growth taken near the TTC strip. Growth taken from the other double layer contained a mixture of C. jejuni and C. coli from which C. jejuni was obtained by selecting some individual colonies on subculture.

All re-isolated and purified strains from the mixtures were re-tested by this screening test.

### Results

Overall 326 strains were tested. One hundred and forty (42.9 per cent) were classified as C. jejuni, 31 (9.5 per cent) as C. coli and 155 (47.5 per cent) as fetus GC. Twenty animals yielded both jejuni/coli GC and fetus GC strains, recovered from 19 PN and one RNBGT plate in which two types of colonies were detected (Figures 3.1 and 3.2). Another 17 dual infections were detected by the screening test but not on the selective plates as follows: twelve mixtures of C. jejuni and fetus GC, four of C. jejuni and C. coli and one of C. coli and fetus GC. Details of Campylobacter spp., groups detected by this screening test are shown in Table 5.7 and the final species identification of all mixtures in Table 5.8.

All jejuni/coli GC strains, typical in respect of growth and morphology, were sensitive to Nal and resistant to C. Most C. jejuni strains (hippurate positive) had variable narrow TTC sensitivity haloes. C. coli strains TTC tolerance tests were



as follows: 16 strains were completely resistant (0 mm) and the rest had variable haloes between 1 up to 6 mm. All fetus GC were resistant to Nal and all but 4 strains were sensitive to C. All fetus GC except one were sensitive to TTC having more than 6 mm of inhibition haloes. Details of these tests will be considered together with the results of the previous scheme.

All 326 campylobacter strains were tested for hippurate hydrolysis. All jejuni/coli GC which had a negative test after 2 hours were considered to be C. coli and all positive strains including those weakly so (Figure 5.1) were considered to be C. jejuni. Twenty three C. jejuni strains gave weak results; when the tests were repeated 18 of them were finally considered as weakly positive but 5 strains became frankly positive after 2 hours. Standard strains had been tested many times and consistently C. jejuni biotype 2 NCTC11392 quickly hydrolysed hippurate and formed glycine detected by formation of a dark purple colour within 10 minutes of the addition of the ninhydrin solution; on the other hand, C. jejuni biotype 1 NCTC11168 generally reacted very weakly or not at all at 10 minutes but was always positive after 2 hours incubation at room temperature. Overall, 152 fetus GC were negative for the hippurate test but traces of colour were recorded in 3 strains which were finally identified as CHI type 1. Comparative readings of <sup>the</sup> hippurate test after incubation for 10 and 120 minutes at room temperature are shown in Table 5.9.

Microscopic observation of carbol-fuchsin smears, appearance and consistency of growth were very useful characteristics to complete the identification of the groups and correlated well

with the other tests. In all cases of doubtful results mixtures of campylobacter groups were discovered. The results were the same as described in the previous biotyping scheme.

### Discussion

In this simplified scheme four tests combined with microscopic and cultural characteristics quickly identified C. jejuni and C. coli and the fetus GC. The use of a few tests left more available time to pay close attention to each individual strain and the work with uncloned strains allowed the detection of "hidden" campylobacter mixtures which otherwise would have not been discovered. As was described in Chapter 4 this screening test combined with the second year procedure led to the isolation of significantly more campylobacters. Twenty dual infections were detected through the re-incubation of previously positive selective plates (Figures 3.1 and 3.2). Thirteen jejuni/coli GC and fetus GC mixtures were detected and identified by means of the screening scheme. In these cases the spreading nature of growth of jejuni/coli GC prevents the isolation of more than one campylobacter in dual infections. For example, C. coli was obtained from two mixtures (Figure 5.9) by subculturing the growth of the layer adjacent to the TTC strip. These were further clear examples that this system of work detected only some of the many mixtures which probably existed, especially as many samples were only cultivated on one selective medium and only one colony per plate characterised.

Strain 379/83-D3258-12 was a mixture of C. coli and CHI type 1. It was first diagnosed as a pure culture of C. coli after the first screening test although it was noted that the typical

cells contained a minority of coarse campylobacters. Due to this characteristic this strain was considered of interest and was re-tested together with the standard strains a further 4 times without different results. Nevertheless, when the 5th screening test was read some few tiny colonies were discovered growing near the Nal disc and when subcultured a typical fetus GC growth was obtained.

Mixtures of C. fetus and CHI may be undetectable either by the screening test, or on primary selective plates or by microscopic observation as they have similar antibio-chemo-sensitivity and colonial and microscopic morphologies. Nevertheless they have been isolated from the same animal as has been described in Chapter 3, Section D (Table 3.9).

Considering these few examples it is evident that detection of mixed cultures may have been incomplete and possibly other mixtures may not have been discovered. Furthermore, as only a few strains were studied in detail it has to be admitted that few "hidden" mixtures of C. jejuni and C. coli could have been missed and in this case the mixture would have been hippurate positive and hence finally identified as C. jejuni. Similar "hidden" mixtures of CHI and C. fetus would have been finally classified as CHI because these mixtures would have been H<sub>2</sub>S-TSI positive (vide infra). During the first year of work the campylobacters were fully cloned before biotyping the proportion of mixtures should have been reduced to a minimum. If such mixtures had occurred in significant numbers it should be expected that C. coli and C. fetus isolations as a proportion of jejuni/coli

GC and fetus GC respectively should have diminished during the second year with respect to the first. Considering only the strains obtained from the surveys, during the first year 46 (28.7 per cent) C. coli were classified out of the 160 jejuni/coli GC strains and during the second year 31 (21.2 per cent) out of 146; these differences are not statistically significant (Yates' corrected  $\chi^2 = 1.909$ ,  $p < 0.20 > 0.10$ ). During the first year 9 (14.5 per cent) C. fetus were classified out of 62 fetus GC strains and during the second year 28 (18.1 per cent) C. fetus out of 155 fetus GC. Probably, therefore the proportion of "hidden" undetected mixtures has been small. This problem of natural mixtures of campylobacters deserves attention because it probably is not restricted to ruminants and strains or species may remain undetected.

### 3) Tests of chemical and antibiotic tolerance - Combined results of the biotyping and screening schemes

Work already described appeared to indicate the existence of separate groups within fetus GC. This study analyses the data of the TTC, Nal and C tolerance tests and compares current bovine campylobacter isolates with standard strains seeking support for such a view. Additionally bovine campylobacter isolates were compared to standard strains in order to know whether differences exist in their sensitivity pattern.

### Materials and Methods

The distance from the edge of growth to the strip or disc was measured with a ruler to the nearest mm (Figures 5.4, 5.5 and 5.6).

These data was recorded for all strains tested including the standard strains each time they were used as controls. In this procedure of work a loopful of growth was suspended in NB-FBP medium (Chapter 2) rather than standardizing the suspension to a turbidity Brown's tube No. 8 as originally described by Skirrow and Benjamin (1980b). This method was tested at the beginning but was found too time consuming for a single worker handling many strains and the alternative was adopted as a quick method after preliminary tests in which the size of the loopful was approximately standardized to yield a suspension of Brown's tube No. 8 opacity.

Occasionally, some strains grew poorly as for instance strain CHI type 1 1393/82 (Figures 5.4, 5.5 and 5.6) and their growth was only obvious in TTC test, in other cases no growth occurred as with 10 "C. fecalis" isolates. Such results were disregarded and in these and similar cases whenever necessary the tests were repeated.

One test for each strain was considered including all the campylobacters isolated from the surveys and the extra strains studied in the screening test. The available data of a number of tests carried out with standard strains were recorded to assess the variability of this method for each individual strain calculating the confidence limits (Downie and Heath, 1974).

## Results

Nal and TTC tests were carried out on 530 strains, 461 strains were tested in the C tolerance test (Table 5.10B). This information

was compared with 89 Nal, 91 TTC and 51 C tests carried out with 8 standard strains (Table 5.10A).

Comparing the results it is noted that C. jejuni bovine and ovine isolates generally have narrower Nal zones of inhibition of growth ( $\bar{x}$  = 9.82 mm) than the two human reference strains C. jejuni biotype 1 ( $\bar{x}$  = 11 mm) and C. jejuni biotype 2 ( $\bar{x}$  = 11.1 mm). Only 42.5 (108/254) and 47.6 per cent (121/254) isolates had Nal inhibition zones within the 99 per cent confidence limit values of the standard C. jejuni biotype 1 and 2 strains respectively, and the rest are asymmetrically distributed with 44.1 per cent (112/254) of the isolates below and only 13.4 and 8.3 per cent above this confidence limit. On the other hand, the Nal inhibition of bovine and ovine C. coli isolates ( $\bar{x}$  = 9.45 mm) are similar to those of the porcine C. coli NCTC11353 standard strain ( $\bar{x}$  = 9.93 mm).

All 194 fetus GC were completely resistant to Nal. The "atypical" fetus GC isolates did not grow in agar for tolerance tests containing FBP (vide infra) or were sensitive to Nal; both standard strains grew and had very similar sensitivity to that of 4 "C. fecalis" and 1 C. sputorum subsp. bubulus which grew on this medium.

Both bovine and ovine isolates and standard strains of the fetus GC were notable more sensitive to TTC than jejuni/coli GC strains (Tables 5.10A and B). C. jejuni isolates were generally more sensitive to TTC ( $\bar{x}$  = 5.72 mm) than C. coli isolates ( $\bar{x}$  = 2.04 mm). The standard porcine C. coli was completely resistant to TTC consistently growing up to the edge of the strip (Figure

5.4) but only 57.1 per cent (44/77) of the bovine and ovine isolates were also completely resistant. On the other hand, only 4.3 per cent (11/254) bovine C. jejuni isolates grew up to the edge of the TTC strip (0 mm).

Comparing the values of Tables 5.10A and B it is noted that the porcine CHI type 1 NCTC11562 standard strain is less sensitive to TTC ( $\bar{x}$  = 9.55 mm) than both bovine CHI type 1 ( $\bar{x}$  = 12.79 mm) or type 2 ( $\bar{x}$  = 12.08 mm) strains. Interestingly, bovine CHI type 1 and type 2 were more sensitive to TTC than bovine C. fetus isolates ( $\bar{x}$  = 10.03 mm, Table 5.10B). In fact, 75.4 per cent (83/110) of the CHI type 1 bovine isolates registered TTC inhibition zones above the 99 per cent confidence limit of the bovine C. fetus isolates, 18.2 per cent (20/110) fell within the limits and 6.4 per cent (7/110) below. Similarly, 68.1 per cent (32/47) of the bovine CHI type 2 isolates had TTC values above this confidence limit, 27.6 per cent (13/47) within and 4.3 per cent (2/47) below.

The standard ovine "C. fecalis" strains as well as the 5 "atypical" fetus GC isolates which grew on FBP agar were sensitive to TTC. The standard strains were more sensitive than the isolates but due to the inhibitory effect of the FBP on the growth of some members of this group this data will be considered later on when tests will be described carried out in media without FBP (Table 5.15).

All 277 jejuni/coli GC strains were completely resistant to C as were 3 standard strains of this group and C. laridis (Tables 5.10A and B). On the other hand, all fetus GC isolates were



sensitive to C except for three CHI type 1, one CHI type 2 and three C. fetus strains; overall only 7 out of 184 fetus GC strains (3.8 per cent) were sensitive to C. Bovine C. fetus isolates were less sensitive to C ( $\bar{x} = 4.63$  mm) than bovine CHI type 1 ( $\bar{x} = 6.67$  mm) or type 2 ( $\bar{x} = 6.24$  mm) isolates. C. fetus and CHI standard strains had similar values although they proved slightly more sensitive than most of the survey isolates (Table 5.10A). "C. fecalis" standard ovine strains were sensitive to C with very similar values to those registered for one C. sputorum subsp. bubulus and 4 "C. fecalis" ovine isolates (Tables 5.10A and B).

#### 4) Fetus GC screening scheme

All fetus GC isolates which had been generically classified and purified from other campylobacters or contaminants and which were later kept frozen were re-classified following a simplified scheme of 3 selected tests. These tests were developed as a result of more detailed tests on a limited number of strains (this Chapter, Section B).

#### Materials and Methods

Hydrogen sulphide production in TSI and Iron medium tests were performed as described previously. Care was taken to prepare, store, inoculate, incubate and interpret these tests strictly and exactly as it was referred to in Chapter 2 and in the introduction to this Chapter. Campylobacter standard strains were used as controls as shown in Table 2.2.



Slide agglutination tests for detection of CHI type 1 strains were carried out with CHI type 1 antisera NCTC11562 obtained from Dr. G.H.K. Lawson, University of Edinburgh, according to the technique described in Chapter 2. Before starting the work with the isolates the sera was tested against all the standard strains. Positive agglutination only occurred with the homologous CHI strain, whilst C. jejuni biotype 1 and 2, C. coli, C. laridis, C. fetus, "C. fecalis" and C. sputorum subsp. bubulus standard strains did not agglutinate.

### Results

In this scheme all strains negative in both H<sub>2</sub>S tests and serologically non-related to the strain CHI NCTC11562 were considered to be C. fetus (Table 5.11). Occasionally, in some tests carried out with C. fetus subsp. fetus NCTC5850 a weak positive H<sub>2</sub>S production was present only on the surface of the slope and never in the butt of the TSI medium. This faint and superficial blackening disappeared after 2-4 hours of exposure to air and was regarded as a negative test (Figure 5.3, bottle No. 2). All 37 C. fetus isolates were completely negative either for H<sub>2</sub>S-TSI test (Figure 5.3, bottle No. 3) or H<sub>2</sub>S-FBP test (Figure 5.2, bottle No. 1).

All campylobacters producing H<sub>2</sub>S from TSI (Figure 5.3, bottle No. 1) and not from Iron medium (Figure 5.2, bottle No. 1) were considered to be CHI (Table 5.11). If they displayed a positive agglutination with antisera CHI NCTC11562 they were arbitrarily classified as type 1 whereas all strains which did not agglutinate were called type 2; 67.9 per cent of the bovine CHI isolates were serologically related with the porcine CHI standard strain

(Table 5.11). CHI type 2, C. fetus, "C. fecalis" or C. sputorum subsp. bubulus were not agglutinated.

All 165 CHI isolates and the standard strain CHI NCTC11562 produced profuse blackening of TSI medium to a small extent in the slope and markedly in the butt (Figure 5.3, bottle No. 1). The CHI NCTC11562 strain and all but 6 CHI isolates did not produce H<sub>2</sub>S from Iron medium; the exceptions were 2 CHI type 1 and 4 CHI type 2 strains. These 6 strains were classified as CHI because they were morphologically and culturally distinct from the "atypical" fetus GC and were differentiated as described in the biotyping scheme.

Finally, all strains which produced H<sub>2</sub>S in both media (Figures 5.2, bottle No. 2 and 5.3, bottle No. 1) did not agglutinate and were morphologically and culturally distinct from the rest, were considered to be "atypical" fetus GC. Catalase positive strains were classified as "C. fecalis" and catalase negative ones as C. sputorum subsp. bubulus (Table 5.11). The same results were obtained with control strains "C. fecalis" 11362 and 11411 and C. sputorum subsp. bubulus NCTC10355.

##### 5) Biotyping of fetus GC from other origins

In general, the biotyping of jejuni/coli GC is relatively straightforward and the classification followed here is comparable to the system used by many other authors. The confused taxonomic state of the fetus GC is different and neither nomenclature nor tests have been agreed. After the development of these biotyping

schemes this new system of work was applied to re-classify a reduced number of fetus GC strains obtained from other investigators and different origins.

#### Materials and Methods

The following tests were carried out: Nal, TTC and tolerance tests, catalase, spread, consistency and aspect of growth, coccal transformation, microscopic morphology, H<sub>2</sub>S production in TSI and Iron media and slide agglutination test with antisera CHI type 1 NCTC11562. C. sputorum subsp. bubulus and "C. fecalis" were not examined for TTC and C tolerance tests. The biotyping criteria was that previously described in other schemes.

Porcine strains: 5 strains obtained from Dr. G.H.K. Lawson, University of Edinburgh, C. fetus (No. 2 and 3, Table 2.1) and "C. coli type I" (CHI type 1, No. 2, 3 and 4, Table 2.1). Two CHI strains obtained from Dr. Gebhart, University of Minnesota (CHI type 1, No. 6 and 7, Table 2.1).

Human strains: 12 C. fetus strains obtained from Dr. I. Heinzer, University of Bern (No. 4 to 11 and 13 to 16, Table 2.1). Strain 12 was not included in the study because it could not be recovered from the freeze dried state.

Bovine strains: 5 C. sputorum subsp. bubulus (strains No. 1 to 5, Table 2.1) obtained from Dr. G.H.K. Lawson, University of Edinburgh and 1 "C. fecalis" (CHI type 1 No. 5, Table 2.1) obtained from Dr. Al-Mashat, University of Glasgow.

#### Results

Following this biotyping scheme, the porcine strains 81-6107 and 81-12235 originally described by Gebhart, Ward, Chang and

Kurtz (1983) as CHI were biotyped as CHI type 1. Porcine strains 614/73-VC, 632/74-A2 and 182/74-VC were also classified as CHI type 1. The bovine strain 75205 originally described as "C. fecalis" by Al-Mashat and Taylor (1980a and 1981) was re-classified as CHI type 1 the strains being resistant to C. All these strains were similar to the standard strain CHI type 1 NCTC11562 (Table 5.12).

Porcine strains 197/73-SI-3 and 1577/78-TSI-5 were classified as C. fetus. All 11 human strains were classified as C. fetus although they differed in some characteristics; all but two strains were resistant to C (3'378/81 and 5'213/81), strain 496/80 was also resistant to TTC (R = 0 mm) and in general they were more resistant to TTC ( $\bar{x}$  = 7.1 mm, standard deviation = 3.85 mm) than the bovine C. fetus strains (Table 5.12).

Bovine strains 551/73-AA4, 584/73-SH1 and 772/73-HF1 were confirmed as C. sputorum subsp. bubulus and 584/73-SH4 was classified as "C. fecalis". These strains were culturally, morphologically and biochemically indistinguishable from the "atypical" fetus group and from the standard "C. fecalis" 11362 and C. sputorum subsp. bubulus NCTC10355 (Table 5.12).

### Discussion

In this study it has been demonstrated that the porcine strains recently described as CHI by Gebhart, Ward, Chang and Kurtz (1983) are indistinguishable from those previously described by Lawson, Rowland and Wooding (1975) as "C. coli type I" from which strain 124/73-A4 NCTC11562 was chosen as a standard representative. Al-Mashat and Taylor (1980a) isolated a strain from lesions of

the gastrointestinal tract of cattle, designated it "C. fecalis" 75205 and later used this strain to experimentally infect calves (Al-Mashat and Taylor, 1981). The re-classification of this strain as CHI type 1 according to this biotyping scheme will allow comparison of the experimental works done by these authors with those of this thesis. This study also makes a contribution to the taxonomy and epidemiology of campylobacters as it has demonstrated that CHI strains are the most common of bovine fetus GC faecal isolates in the surveys described in this thesis (Chapter 4, Section B), that such strains have been previously found in bovines and the same species is commonly found in the intestinal tract of pigs.

CHI strains are commonly recovered from cattle and pigs and it was considered of interest to ascertain whether these species could eventually be also isolated from humans. Heinzer (1983) isolated C. fetus from patients with febrile enteritis and found that these 12 campylobacters behaved variably in temperature tolerance tests as 9 strains grew at both 25°C and 42°C and 3 grew typically only at 25°C (Heinzer, Personal Communication, 1983). As CHI strains have also variable growth at different temperatures these human campylobacters were considered of interest for the study. The presence of CHI in humans was not demonstrated but interestingly these C. fetus strains were atypical in their C and TTC resistance, other characteristics were the same as described for bovine C. fetus. The porcine C. fetus were indistinguishable from the bovine strains.

The 4 bovine campylobacters isolated from bull's preputial

washings by Lawson, Rowland and Wooding (1975) were identical in these tests to the bovine faecal isolates provisionally named "atypical" fetus GC and to the standard "C. fecalis" and C. sputorum subsp. bubulus. All these strains were sensitive to NaI when the test was carried out with tolerance agar containing FBP, did not swarm, had a sticky and adherent growth on CBA plates, had a variable coccal transformation test, had the typical microscopic morphology of the group and produced H<sub>2</sub>S from both TSI and Iron media. These strains will be described later in a detailed study on other characteristics of this group.

### General Discussion

#### Campylobacter nomenclature and taxonomy

Due to the doubtful taxonomic position of the members of the fetus GC in this study it was necessary to use a provisional taxonomic nomenclature to describe the members of this group. The recent CHI denomination of Gebhart, Ward, Chang and Kurtz (1983) to designate campylobacters which are closely related to C. fetus but which differ in producing H<sub>2</sub>S from insensitive media is a provisional designation adopted in this study, it is not known yet if this group constitutes a separate species or merely a C. fetus subspecies. Bovine CHI serologically related to the standard porcine CHI strain were arbitrary designated type 1 and those not related type 2. As was noted in Chapter 1, the subspecies of C. fetus, C. fetus subsp. fetus and C. fetus subsp. venerealis are 100 per cent genetically homologous (Belland and

Trust, 1982; Harvey and Greenwood, 1983). For that reason in this study the name C. fetus without subspeciation, was adopted. As the intestinal tract was the source of the strains they could be assumed to be C. fetus subsp. fetus.

C. sputorum subsp. bubulus (Prévot, 1940) has been defined as a catalase negative campylobacter, campylobacters which are similar but differ only in being catalase positive are called "C. fecalis" (Firehammer, 1965). Smibert (1974) described "C. fecalis" as a different species not related to any other group of campylobacters but this scheme has not been officially approved (Skerman, McGowan and Sneath, 1980). Contradictory results have been published on DNA relatedness data. For instance, Owen and Leaper (1981) found that strain A125/81, an organism conforming to the description of "C. fecalis", had closer affinities to C. fetus and C. concisus than to other campylobacter species. On the other hand, Harvey and Greenwood (1983) compared 5 "C. fecalis" strains received from Firehammer with one C. sputorum subsp. bubulus strain and found that these two species are 75 per cent genetically related. This shows that confusion in the taxonomy of this group is widespread because if campylobacters are classified as "C. fecalis" mainly because they are catalase positive,  $H_2S$  producers in insensitive medium and  $43^{\circ}C$  growers both CHI and "C. fecalis" strains could enter in this category. Probably, the "C. fecalis" described by Owen and Leaper (1981) is in fact CHI and this could explain why such different results have been obtained. In the biotyping scheme developed in this study a combination of biochemical tests with observation of microscopic and cultural



characteristics have proved very useful to reliably differentiate "C. fecalis" and CHI strains. "C. fecalis" is phenotypically similar and genetically related to C. sputorum subsp. bubulus (Harvey and Greenwood, 1983) and CHI is phenotypically similar and genetically related to C. fetus (Ursing, Sandstedt and Hansson, 1984). Walder, Sandstedt and Ursing (1983) described a group of eight Nal-resistant, hippurate negative isolates of porcine and bovine origin which according to Skirrow (1983) are similar to or perhaps even the same as CHI. This group of campylobacters is genetically heterogeneous and forms a separate group of campylobacters closely related to C. fetus (Ursing, Walder and Sandstedt, 1983). They did not produce  $H_2S$  from TSI (Walder, Sandstedt and Ursing, 1983; Sandstedt, Ursing and Walder, 1983) under microaerobic conditions (5 per cent oxygen and 7 per cent carbon dioxide in nitrogen) but under anaerobic conditions (rich hydrogen atmosphere) a blackening of the upper part of the TSI was produced (Ursing, Sandstedt and Hansson, 1984).

#### Hippurate hydrolysis

Considering only those strains which were isolated in the surveys, hippuricase activity was detected in 71.3 and 78.7 per cent of the jejuni/coli strains classified by means of the biotyping and screening schemes, respectively. After 2 hours of incubation at room temperature weak reactions were recorded in 2.5 and 13 per cent of the jejuni/coli GC strains classified by the biotyping and screening schemes, respectively. More weak reactions were detected in strains examined by the screening test. These differences could be due to a different proportion of species



recovered during the two years of work or to the different procedures for the test. Two combinations of temperature, atmosphere and time were used for incubation of the inoculum,  $43^{\circ}\text{C}$ - $550\text{H}_2$  for 18 hours and  $37^{\circ}\text{C}$ - $650\text{H}_2$  for 2 days for the biotyping and screening schemes, respectively. Incubation at  $37^{\circ}\text{C}$  may be a better method for detection of weak hippuricase activity than incubation at  $43^{\circ}\text{C}$ .

Using a procedure in which the inoculum was grown at  $35^{\circ}\text{C}$  or  $37^{\circ}\text{C}$  and re-incubating the tubes or bottles after the addition of ninhidrin for 10 minutes before the final reading it was found that 81 and 84 per cent of the jejuni/coli GC strains examined hydrolysed hippurate (Hébert, Hollis, Weaver, Lambert, Blaser and Moss, 1982; Luechtefeld and Wang, 1982). In this work it was demonstrated that incubating the bottles for 10 minutes at room temperature after the addition of ninhidrin is insufficient as 23.3 per cent of 146 jejuni/coli strains tested at both incubation periods were negative at 10 minutes but positive at 2 hours.

Harvey and Greenwood (1983) compared hippuricase activity and DNA-DNA hybridization and found that one out of 22 hippurate negative jejuni/coli GC strains examined was more closely related to C. jejuni than to C. coli. This strain was phenotypically C. coli but genetically 85 per cent related with C. jejuni and only 38 per cent to C. coli. As was found in this work intermediate and dubious reactions have been reported and the necessity of re-testing some strains recommended (Luechtefeld and Wang 1982). This work has shown that some strains consistently gave a weakly positive hippurate test whereas other strains yielded different results

in separate tests.

CHI, C. fetus, "C. fecalis" and C. sputorum subsp. bubulus were all hippurate negative, except for 3 CHI strains which were weakly positive and which represent less than 2 per cent of the fetus GC strains investigated. This work confirmed previous observations that C. fetus subspecies, C. sputorum subspecies and "C. fecalis" do not hydrolyse hippurate (Harvey, 1980; Luechtefeld and Wang, 1982; Hébert, Hollis, Weaver, Lambert, Blaser and Moss, 1982) and contributed considerable new information on CHI bovine and porcine strains of which a few strains only have been previously examined in this test (Gebhart, Ward, Chang and Kurtz, 1983).

#### Hydrogen sulphide production from Iron and TSI media

The H<sub>2</sub>S-FBP production test has proved useful in the identification of "C. fecalis" and C. sputorum subsp. bubulus strains which have been described as strong H<sub>2</sub>S producers (Florent, 1953; Firehammer, 1965). A similar test was proposed some years ago to differentiate C. fetus from C. sputorum subsp. bubulus, it consisted of a basal Brucella agar medium plus 0.1 per cent FeSO<sub>4</sub>.7H<sub>2</sub>O which was heavily inoculated and then incubated at 37°C in candle jars (Ringén and Frank, 1963). The medium used here contained 0.05 per cent of each FeSO<sub>4</sub>.7H<sub>2</sub>O, sodium metabisulphite and sodium pyruvate. All C. fetus and 96.4 per cent of CHI strains were negative and all "C. fecalis" and C. sputorum subsp. bubulus were positive H<sub>2</sub>S-FBP producers. Further work might reduce the sensitivity of the method in order to increase its specificity for C. sputorum subsp. bubulus and "C. fecalis" strains

by testing different combinations of the FBP components. For instance, recent studies of Jorgensen (1983) with jejuni/coli GC demonstrated that the addition of sodium metabisulphite increased the sensitivity of this test and maximum blackening appeared in a few hours but in contrast without its addition the test was positive only after 1 or 3 days of incubation at 37°C.

The TSI medium was useful to differentiate CHI from C. fetus strains. The sensitivity of this medium was increased considerably when incubated in a rich hydrogen atmosphere and when recently prepared medium was used. The described superficial faint blackening of C. fetus subsp. fetus NCTC5850 (Figure 5.3, bottle No. 2) was detected only when fresh medium was employed. Positive H<sub>2</sub>S-TSI campylobacters have not only to be able to produce H<sub>2</sub>S but also to grow in deep stabs. Campylobacters such as C. sputorum subsp. bubulus, "C. fecalis" and CHI which require levels of 3 or less per cent of oxygen are probably able to grow in deep stabs more successfully than other campylobacters such as C. fetus which require 6 per cent of oxygen. This test is useful not only because it measures the ability to produce H<sub>2</sub>S but also a reduced oxygen requirement which may be expected to be correlated with genotypical differences of some groups. Further information can be obtained from this test which detects acid or gas production from carbohydrates (dextrose, lactose, sucrose); none of 217 fetus GC bovine isolates examined nor any of the other fetus origin GC strains studied showed any acid or gas formation from carbohydrates.

#### Tests of chemical and antibiotic tolerance

Combining Nal and C sensitivity tests on FBP tolerance agar three groups of campylobacters can be distinguished: jejuni/coli GC were all without exception sensitive to Nal and resistant to C; CHI and C. fetus were resistant to Nal and generally sensitive to C with a few strains resistant and "C. fecalis" and C. sputorum subsp. bubulus were sensitive to both, although the Nal sensitivity zones were narrow and many of these strains did not grow on the basal medium. In general, this work confirmed the usefulness of these tests to identify campylobacter groups but they should be used only together with microscopy and cultural characteristics. Exceptions have been found and show that these tests are not infallible; in this work C resistant fetus GC were isolated and other authors have encountered a Nal resistant C. jejuni human isolate which was genetically confirmed (Ursing, Walder and Sandstedt, 1983). Besides, Holländer (1982) found that 15 per cent of 256 jejuni/coli GC isolates were resistant to Nal (MIC 40 µg/ml); these striking differences could be due to real geographical variations among jejuni/coli GC or more probably to the use of a different biotyping system (Holländer, 1982) which classifies some fetus GC which grow at high temperature as jejuni/coli GC.

For clinical identification of campylobacter groups it is preferable that results should be based on a zone or no-zone phenomenon, so that actual zone sizes need not be measured. In this work by measuring the inhibition zones it was found that in general, bovine C. fetus isolates were less sensitive to C than CHI strains although the contrary was found for the standard

strains. Skirrow and Benjamin (1980b) obtained mean values of Nal sensitivity zones of  $\bar{x} = 11.40$ ,  $S = 1.74$  with 1120 jejuni/coli GC strains, mostly of human origin which are higher than those obtained in this work with bovine isolates (Table 5.10B). In the work of this thesis similar values were obtained in a number of tests with two human C. jejuni standard strains (Table 5.10A) demonstrating that the methodology of both works is comparable. It has to be concluded that bovine C. jejuni strains are generally less sensitive to Nal than human C. jejuni which may have epidemiological implications.

In agreement with the results of other workers (Skirrow and Benjamin, 1980b; Luechtefeld and Wang, 1982) most of the jejuni/coli GC strains studied were resistant to TTC whereas nearly all fetus GC were sensitive (more than 6 mm). Confirming previous observations (Véron and Chatelain, 1973) it was found in this study that C. coli was generally more resistant to TTC than C. jejuni. It was found that, despite individual variations, bovine CHI isolates were more sensitive to TTC than C. fetus ones. In fact, 73.2 per cent (115/157) of the bovine CHI isolates had TTC inhibition zones above the 99 per cent confidence limit of bovine C. fetus isolates, another characteristic which supports the view that CHI and C. fetus may be separate species. The few porcine CHI studied were less sensitive than the bovine strains. Skirrow and Benjamin (1980b) studied 42 C. fetus subsp. fetus from cattle (10 strains), sheep (21 strains), pigs (2 strains) and man (9 strains) isolated from the intestinal and genital tracts and found that they were more sensitive ( $\bar{x} = 13.7$ ,  $S = 2.77$ )

than the bovine C. fetus isolates of this work (Table 5.10B). They found that "atypical" C. fetus strains (this group may have included some CHI strains and C. fetus which grew at 43°C) were equally sensitive although only 16 strains of this group were studied.

#### Temperature tolerance tests

This test was useful only as a complementary characteristic of the jejuni/coli GC which consistently grew at 43°C but not at 25°C. The fetus GC showed a diversity of results which made this test useless for taxonomic purposes (Table 5.6). Interestingly the few bovine C. fetus strains examined grew at 25°C but not at 43°C. CHI strains were variable in their tolerance of temperature as strains which grew at both temperatures, at only one of them or even at none of them and only at 37°C were encountered. "C. fecalis" and C. sputorum subsp. bubulus grew at 43°C but not at 25°C although when the test was first performed with a not so accurate system of control of the low temperature which allowed oscillations of  $\pm 0.5^{\circ}\text{C}$ , some strains of this group grew at 25°C.

The jejuni/coli GC are also known as thermophilic campylobacters but this term has not been used in this thesis because it is not a characteristic exclusive of this group of campylobacters. The only group in which the temperature tolerance test gave consistent results was the jejuni/coli GC but this group can be identified by other tests. It would be interesting to know whether other bovine C. fetus grow at 25°C but not at 43°C. It is concluded that these tests are not practical or useful for taxonomic identification of ruminant campylobacters.

#### Catalase test

In this work differences in the peroxidase activity of some strains were noted. Five C. jejuni biotype 1 were weakly positive on repeated tests. CHI and C. fetus strains were in all cases strongly positive. Only three atypical fetus GC were negative and were therefore classified as C. sputorum subsp. bubulus the rest were strongly positive and were classified as "C. fecalis" except for one strain which was weakly positive.

Variations in the amount of peroxidase production have been described and Firehammer and Lovelace (1961) cited that campylobacters which produce weak or strong catalase reactions but have other physiological characteristics of C. sputorum subsp. bubulus are commonly found in the genital tract of sheep and should be tentatively placed in the former species. Later, Firehammer (1965) isolated similar strains from the intestinal tract of sheep and proposed to designate them "C. fecalis". In this work, catalase test provisionally separated "C. fecalis" and C. sputorum subsp. bubulus although it is not known if they should be considered the same species with variable catalase activity or if they are a closely related different species.

#### Cultural and microscopic characteristics

There are so few reliable tests to differentiate campylobacter species and exceptions are<sup>60</sup> commonly found that observation of cultural and microscopic characteristics play an important role in the practical identification of the groups. Combining these characteristics three groups can be clearly distinguished:

1) jejuni/coli GC: characteristic spread and metallic sheen of growth, small and thin cells of small wavelength and amplitude



and rapid coccal transformation.

- 2) fetus GC: weak or no spread with matt aspect of growth, big and predominantly long cells with long wavelength and marked amplitude and negative coccal transformation.
- 3) "atypical" fetus GC: no swarming with characteristic sticky adherent growth, predominantly short comma cells with straighter undulations and variable coccal transformation.

#### Proposal for a simple biotyping scheme

All the tests used in this work except temperature tolerance tests were useful in the classification of the species although tests should not be used alone as they are effective only when used in combination with the others. The following tests form the basis of the scheme for differentiation of campylobacter groups: growth aspect; microscopic morphology; coccal transformation and NaI, TTC and C tolerance. For differentiation between C. jejuni and C. coli: hippurate test. For differentiation between C. fetus, CHI and "atypical" fetus GC: H<sub>2</sub>S production in Iron and TSI media. For differentiation between C. sputorum subsp. bubulus and "C. fecalis": catalase test. Recommended modifications for the aforementioned procedures: 1) campylobacter strains which are to be tested for hippurate test should be grown only at 37°C on CBA plates; 2) a study of the sensitivity of Iron medium should be undertaken in order to make it more specific for the diagnosis of "C. fecalis" and C. sputorum subsp. bubulus.

#### The presence of multiple campylobacter strains in faeces

The work with uncloned isolates showed that<sup>3</sup> mixed campylobacter flora is a natural common phenomenon in ruminants. Some mixtures



were detected either on the selective media or by the tolerance test used but the further discovery of subtle mixtures proved that this system is not completely efficient. One of the objectives of this work was to assess the possible importance of jejuni/coli GC in calf diarrhoea. For this purpose fully satisfactory selective media were available and it is believed that the results presented for this species in general represent the situation in the field. We may have less confidence in respect of the other campylobacter species that the media available were optimal, therefore results involving these species may not be fully representative of all the isolations that could be obtained. It is also possible that further Campylobacter spp. exist which have not as yet been isolated and the assessment of the association between campylobacters and disease is certainly not exhausted.

## CHAPTER 5: SECTION B

### FURTHER BIOTYPING AND CHARACTERIZATION OF CAMPYLOBACTERS

In this section, some biochemical, serological and morphological studies carried out on selected campylobacter strains will be described. Some of these investigations were performed with the purpose of finding new tests which might be useful for routine classification of campylobacters, in particular the fetus GC and to develop a screening scheme for this group. Other studies were carried out in order to provide additional information about tests which have been in use for routine biotyping but where the mechanism of action was not known.

#### 1) Fetus GC taxonomic studies

This work was carried out with 40 representative campylobacter isolates and the results formed the basis for the scheme used in Section A for biotyping fetus GC.

#### Materials and Methods

Campylobacter strains: 40 selected representative isolates (Table 5.13), 25 strains from cattle and 15 from sheep.

The standard strains used were: C. fetus subsp. fetus NCTC5850, "C. fecalis" 11392 and 11411, CHI type 1 NCTC11562 and on occasion C. sputorum subsp. mucosalis NCTC11000. Unless otherwise stated all non-sputorum strains were used in the tests.

Other strains: Acinetobacter anitratus NCTC7844 and Acinetobacter lwoffii 1426/81 were used as negative controls for nitrite reduction tests in which they were incubated aerobically and  $650H_2$  at  $37^{\circ}C$ , for the same period as described for campylobacters.

Staphylococcus aureus NCTC6571 and Staphylococcus epidermidis (NN) were respectively used as positive and negative controls for MG-DNase tests.

Coccal transformation, nitrite reduction, catalase test, growth at 25°C and 43°C were performed as described in Chapter 2. Comparative growth at 37°C-650H<sub>2</sub> and 37°C-650N<sub>2</sub> was carried out in CBA plates incubated for 18 hours. Delayed coccal transformation was read after 8 and 14 days of exposure of the plates to air. MG-DNase agar was prepared as described in Chapter 2 and the test was carried out and interpreted according to Hébert, Hollis, Weaver, Lambert, Blaser and Moss (1982); final reading was done after the 3rd day of incubation at 37°C-650H<sub>2</sub>.

Nal, TTC and C tolerance tests were performed as previously described but agar with and without the addition of FBP compound was used in comparative tests. Tolerance tests on agar without FBP were carried out on all 5 standard strains, and 24 selected strains.

Slide agglutination tests were carried out with antisera CHI type 1 NCTC11562 as previously described (Chapter 2).

## Results

The 18 characteristics defined by the tests were used to classify the campylobacter isolates and standard strains.

Grouping was carried out using a simple numerical method. Using this system it was found that C. fetus subsp. fetus NCTC 5850 and CHI NCTC11562 shared 78 per cent of the characteristics examined, CHI NCTC11562 and "C. fecalis" 11392 and 11411 61 per cent, C. fetus subsp. fetus NCTC5850 and "C. fecalis" 11392 and

11411 50 per cent and the "C. fecalis" standard strains were identical (Table 5.14A). All bovine campylobacter isolates were more related to both CHI and C. fetus subsp. fetus than "C. fecalis" standard strains (Table 5.14B) whereas all ovine strains were found to be similar to the standard "C. fecalis" (Table 5.14C).

All bovine isolates shared 10 common characteristics and were subdivided into six groups by their relatedness level to standard strains by means of the remaining 8 differential characteristics (Table 5.14B). Group I was composed of 6 isolates which were indistinguishable from the standard CHI strain. Group II was composed by 7 isolates which shared all characteristics but different agglutinating antigens with the standard CHI strain, and 3 isolates which differed from the standard CHI strain only by a negative MG-DNase test. Group III was composed of 3 strains which were more related to CHI than any of the other standard strains but were atypical in other characteristics such as H<sub>2</sub>S production on Iron medium or TTC and C resistance. Group IV was an intermediate group which was similarly related to both CHI and C. fetus subsp. fetus standard strains. Group V was composed by strain No. 5 which was identical to C. fetus subsp. fetus and group VI was composed by 3 isolates related to C. fetus subsp. fetus but differing in being DNase producers.

Ovine isolates constituted an homogeneous group sharing 94 to 83 per cent of the characteristics of "C. fecalis" standard strains. The isolates shared 15 common characteristics and could be separated into six closely related groups by means of catalase,

coccal transformation and growth in FBP agar (Table 5.14C).

All strains but ovine isolates No. 28, 29, 33 and C. sputorum subsp. mucosalis standard strain were catalase positive; strain 31 reacted weakly (Table 5.15).

All isolates and standard strains reduced nitrites; after 15 days of incubation all but bovine No. 14 and ovine isolate No. 32 were positive; these two isolates reduced nitrites when the test was read after further 15 days of re-incubation. Both Acinetobacter spp. controls were negative when incubated either aerobically or microaerophilically; it was nevertheless noted that bottles incubated aerobically displayed an intense red colour (negative test) whereas the microaerophilic ones formed a pale red tinge (still clearly negative) when the nitrite reagent was added. Aerobic and microaerophilic un-inoculated control bottles were both consistently negative when the reagent was added after 15 or 30 days of incubation.

All campylobacters grew at 37°C-650H<sub>2</sub> and all, but C. sputorum subsp. mucosalis NCTC11000, grew at 37°C-650N<sub>2</sub>.

All ovine isolates grew at 43°C and failed to grow at 25°C, although very weak growth at 25°C was detected in strains 27, 33 and 34 (Table 5.15). All groups V and VI bovine isolates related to C. fetus subsp. fetus (Table 5.14B), grew at 25°C and failed to grow at 43°C. The temperature tolerance test for bovine isolates of groups I, II, III and IV (Table 5.14C) was variable: 19 isolates grew either at 25°C or 43°C but 10 strains grew weakly at 43°C, one grew at 25°C but not at 43°C (No. 24), one did not grow either at 25°C nor at 43°C (No. 3) and the CHI type 1 NCTC11562 grew at

43°C but very weakly at 25°C.

The standard coccal transformation test after 6 days of oxygen exposure was negative for all bovine isolates, the standard C. fetus subsp. fetus and CHI strains. After 8 days of oxygen exposure only two bovine isolates of group I strains, No. 1 and 12, produced a few coccal bodies, the rest of the bovine isolates and standard strains were negative (Table 5.15). After 14 days of oxygen exposure these 2 and further 7 strains (No. 1, 3, 4, 5, 7, 8, 12, 15 and 16) had formed 50 per cent or less coccal bodies, the rest were negative with very few occasional coccal forms. Delayed coccal transformation results of ovine isolates were different. After 6 or 8 days of oxygen exposure isolate No. 27 and both "C. fecalis" standard strains showed complete coccal transformation, isolates had approximately 50 per cent of coccal forms (No. 28, 30 and 39) and the rest were negative. After 14 days of oxygen exposure the ten isolates (No. 27, 28, 29, 30, 31, 33, 34, 35 and 36) and both "C. fecalis" standards showed complete coccal transformation test, 2 isolates (No. 37 and 39) had approximately 50 per cent or less coccal forms, and the remaining 4 strains (No. 26, 32, 38 and 40) had no coccal forms. All ovine isolates showed in carbol-fuchsin smears for delayed coccal transformation the campylobacters surrounded by an amorphous substance, tinged very faintly by the fuchsin, and provisionally called "M" substance (Table 5.15). This substance was not observed in the "C. fecalis" standard strains and after 14 days of oxygen exposure was no longer visible in some strains. Finally, the standard strain C. sputorum subsp. mucosalis did not

form coccal bodies after 14 days of oxygen exposure.

Most bovine strains produced DNase (13 strains, 62 per cent), 4 strains (19 per cent) reacted very weakly and the other 4 were negative; the standard CHI strain reacted very weakly (Table 5.14). One bovine isolate of group V did not produce DNase, two isolates of group VI produced this enzyme very weakly and one isolate of this group was frankly positive; the standard C. fetus strain did not produce detectable DNase. In all ovine isolates and standard "C. fecalis" strains but one, strongly positive MG-DNase tests were recorded; the exception was strain No. 26 which reacted weakly. C. sputorum subsp. mucosalis NCTC11000 did not produce detectable DNase (Table 4.15).

Nine bovine and all 15 ovine isolates and the 5 standard strains were compared for Nal, TTC and C tolerance tests with agar with and without added FBP. Similar results were obtained in both media in all strains except for ovine isolates (Table 4.15). These last two species either did not grow on FBP agar or if they did they generally grew poorly and were sensitive to Nal. In absence of FBP they all grew and were consistently resistant to Nal.

### Discussion

In this study representative bovine and ovine fetus GC strains were compared with a porcine CHI, bovine C. fetus subsp. fetus and ovine "C. fecalis" standard strains. It was found that bovine strains were related to CHI or C. fetus subsp. fetus strains whereas the ovine isolates were a more homogeneous group closely related to "C. fecalis"



By means of the taxonomic system used in this work it was evident that CHI and C. fetus subsp. fetus standard strains are phenotypically related sharing 78 per cent of the characteristics examined. When this numerical method was applied to classify the bovine isolates (Table 5.14B) it was possible to identify group I as CHI isolates and groups II and III as CHI-like organisms because they were more related to CHI than C. fetus subsp. fetus. Similarly groups V and VI were respectively classified as C. fetus and C. fetus-like organisms. Group IV was composed of two isolates which shared 83 and 89 per cent of the characteristics studied with both CHI and C. fetus subsp. fetus strains; these two strains produced  $H_2S$  from TSI and for that reason were provisionally identified as CHI but may merely form an intermediate group.

The ovine strains were all similar and related (83 - 94 per cent) to ovine "C. fecalis" standard strains (Table 5.14C). They were clearly different from the porcine CHI (50 - 67 per cent) and bovine C. fetus subsp. fetus (39 - 56 per cent). Minor variations within "C. fecalis" strains were distinguished by means of FBP growth, coccal transformation and catalase test. The ability to grow on FBP agar for tolerance test is a relative method of differentiation as all ovine isolates and "C. fecalis" standard strains which grew did so weakly. The differences in the coccal transformation were related to the presence of an "M" substance (vide infra) characteristic which might justify further examination. One strain (No. 31) had a consistently weak peroxidase activity and constitutes an example of a campylobacter

of this group of intermediate activity.

Gebhart, Ward, Chang and Kurtz (1983) described that 4 of their porcine CHI isolates were not able to reduce nitrites and proposed to use this test to differentiate these strains from C. sputorum subsp. mucosalis strains which reduced nitrites. In this study, all bovine CHI isolates reduced nitrites and this test was found useless for taxonomic purposes as also all other fetus GC studied were positive. After this investigation nitrate and nitrite tests were carried out with Gebhart's porcine CHI 81-6107 and 81-12235 strains and Dr. Lawson's porcine CHI NCTC 11562 and 632/74-A2-VC strains (Table 2.1) using the method of this work; it was found that all 4 strains reduced both nitrites and nitrates after 8 days of incubation at  $37^{\circ}\text{C}$ -550H<sub>2</sub> with media containing either 0.001 or 0.01 per cent of NaNO<sub>2</sub> or 0.01 per cent of KNO<sub>3</sub> (Dr. G.H.K. Lawson, Personal Communication, 1984).

Nitrate test has been <sup>the</sup> subject of controversy in campylobacter studies since the original porcine nitrate negative Vibrio coli strain described by Doyle (1944). The results of this work and of Dr. Lawson contradict the findings of Gebhart, Ward, Chang and Kurtz (1983) but the technique used by these investigators differed from that employed in this laboratory. These authors used semisolid thioglycollate medium with as much as 1 per cent of KNO<sub>3</sub> and incubated at  $37^{\circ}\text{C}$  for only 7 days in an atmosphere containing 7 per cent of H<sub>2</sub>, 6 per cent of O<sub>2</sub>, 7 per cent of CO<sub>2</sub> and the rest N<sub>2</sub>. Differences in medium, incubation time or atmosphere may be responsible for these divergent results. In this study, negative and un-inoculated controls confirmed

that positive campylobacter nitrite reduction results are true. Different and contradictory results of these tests indicate that careful and detailed standardization of the conditions are required for future work on campylobacter nitrate and nitrite reduction determinations.

None of the campylobacter isolates or standard strains, except C. sputorum subsp. mucosalis, showed hydrogen dependence as all grew in presence of either H<sub>2</sub> or N<sub>2</sub> atmospheres. The standard C. sputorum subsp. mucosalis grew only in presence of H<sub>2</sub> as described by Lawson, Leaver, Pettigrew and Rowland (1981) and was used as a biological control for this test.

The temperature tolerance results were not different from those reported previously in the biotyping scheme: bovine, CHI and CHI-like strains were variable, bovine C. fetus and C. fetus-like grew at 25°C but usually not at 43°C and ovine isolates grew at 43°C and generally not at 25°C.

A few coccal forms developed after 8 days of oxygen exposure in some CHI and C. fetus strains showing that reading after 6 days of oxygen exposure of the plates is the optimum for this test as recommended by Karmali, Allen and Fleming (1981). The main aim of this work on delayed coccal transformation was to study in more detail the behaviour of ovine strains. This group of campylobacters has been found to have variable coccal transformation after 6 days of oxygen exposure. Similar results were obtained after 8 days after which some strains transformed completely, others partially and others did not produce coccal forms. After 14 days more strains had developed coccal forms.

It was noted that the amorphous "M" substance was detected more frequently in strains that did not transform and was absent from transformed cultures. This group of "atypical" fetus GC alone had a sticky consistency, mucoid growth and a tendency to form filaments when touched with a Kolle loop. It may be that this "M" substance is related with the consistency of the growth and exerts a protective effect on the cells preventing the formation of coccal forms.

Hébert, Hollis, Weaver, Lambert, Blaser and Moss (1982) applied the MG-DNase test of Smith, Hancock and Rhoden (1969) for the study of campylobacters and found that C. sputorum subsp. bubulus and "C. fecalis" strains hydrolysed DNA whereas C. fetus and C. sputorum subsp. mucosalis did not. In this work it was found that ovine C. sputorum subsp. bubulus and "C. fecalis" generally produced a relatively strongly positive MG-DNase test; bovine CHI and C. fetus strains had a variable activity some strains being frankly positive, others weakly positive and others negative; the only C. sputorum subsp. mucosalis strain tested was negative. One of the problems of this test is the difficulty in discriminating between a negative and a weakly positive test. Staphylococcus aureus NCTC6571 produced a very strong DNase activity evidenced by the formation of a colourless agar halo which spread out 8-10 mm from the macrocolony. Even the strongest DNase producing "C. fecalis" strains only produced haloes which did not spread more than 2 or 3 mm from the macrocolony after 2 or 3 days of incubation. A campylobacter was considered negative if after 3 days of incubation the macrocolony had a green-blue colour

and after removal of the growth no clearing of the underlying agar was found. This test needs to be modified to make it more sensitive for the detection of the weak DNase activity of campylobacters. It was found that generally "C. fecalis" and C. sputorum subsp. bubulus produced stronger DNase activity than CHI or C. fetus although it was technically impossible to accurately discriminate these differences.

It had been found that some "C. fecalis" and C. sputorum subsp. bubulus strains did not grow on agar containing FBP. In this chapter comparative Nal, TTC and C sensitivity tests on agar with and without FBP on CHI, C. fetus, "C. fecalis", C. sputorum subsp. bubulus and C. sputorum subsp. mucosalis strains are described (Table 5.15). It was found that "C. fecalis" and C. sputorum subsp. bubulus strains either were unable to grow or grew weakly in agar containing FBP; interestingly, those which grew in FBP were sensitive to Nal but when the test was performed on agar without FBP they were frankly resistant. All CHI, C. fetus and C. sputorum subsp. mucosalis strains always grew in presence of FBP and were resistant in agar containing or lacking of FBP. The inversion of the nalidixic acid resistance (Nal-Inv) was considered of importance and of taxonomical value for differentiation and recognition of "C. fecalis" and C. sputorum subsp. bubulus from other fetus GC. The phenomenon of the FBP induced inversion of the Nal resistance on "atypical" fetus GC will be studied in detail later on (vide infra).

Characterisation of the strains of fetus GC divided them into a number of taxons, bovine groups I, II, III and IV were considered

to be CHI and bovine groups V and VI C. fetus. All ovine isolates were considered to be "C. fecalis" (catalase positive) or C. sputorum subsp. bubulus (catalase negative). It is evident that any simplified fetus screening scheme can fail to detect groups with intermediate characteristics, such as group IV, which were provisionally classified as CHI strains.

The lack of suitable tests to differentiate and identify campylobacters is notable. Some tests such as nitrite reduction or hydrogen dependent growth were useless because all strains tested gave similar results. Others, such as MG-DNase may be useful but are not sensitive enough for campylobacter strains. Temperature tolerance tests are time consuming and yield variable results with strains otherwise apparently similar. The H<sub>2</sub>S production tests were considered one of the most appropriate available techniques for identification of fetus GC.

## 2) Serological relationship between bovine and porcine CHI strains

In the surveys of this thesis the isolation of 165 CHI strains from bovines has been reported, of these 67.9 per cent were serologically related to the porcine CHI NCTC11562 by means of a slide agglutination test (Tables 5.12 and 5.13). Later, a few bovine and porcine CHI strains from other origins were studied and they were also found to be related to the porcine CHI NCTC 11562. In this thesis the CHI strains which agglutinated in a slide test with the CHI antisera NCTC11562 were arbitrary classified as type 1 and the non-agglutinating ones as type 2.

The purpose of this work was to amplify slide agglutination test results by performing a more detailed study on some CHI type 1 strains.

#### Materials and Methods

Plate agglutination tests were carried out on 35 CHI type 1 strains from different origins (Table 5.16A). Absorption of CHI antisera was performed with the homologous NCTC11562 and 213/82-B293-19 CHI type 1 bovine strains. The last strain was used in experimental disease studies in gnotobiotic lambs (Chapter 6).

Preparation of "OH" antiserum NCTC11562 and "OH" CHI antigens and the methods and interpretation of plate agglutination and absorption tests have been described in Chapter 2. The titre was considered the last dilution at which agglutination was present at 2 or more points of an arbitrary scale of 1 to 4.

#### Results

Plate agglutination results are shown in Table 5.16A. The CHI antiserum NCTC11562 had a titre of 1/1280. Ten other CHI type 1 strains had also a titre of 1/1280, 5 strains 1/640, 11 strains 1/320, 4 strains 1/160, 1 strain 1/80 and 3 strains which had agglutinated on the slide test were negative at 1/40. The porcine CHI type 1 strain 81-6107 (Gebhart, Ward, Chang and Kurtz, 1983) had a titre of 1/160 and the porcine 632/74-A2-VC (Lawson, Rowland and Wooding, 1975) and the bovine 75205 (Al-Mashat and Taylor, 1980a and 1981) had a titre of 1/320.

Absorption has confirmed that the two strains tested share some superficial antigens (Table 5.16B). Strain NCTC11562 probably has more than one superficial factor from which some are common



to strain 213/82-B293-19 but other are different. Absorption of NCTC11562 serum by the homologous strain removed activity from bovine strain 213/82-B293-19 indicating the specificity of the reaction. Antibody left after absorption of the serum with strain 213/82-B293-19 indicates the differences between the strains.

### Discussion

These results showed that CHI strains from either bovine and porcine origin share not only similar biochemical and morphological characteristics but also some superficial common antigens. The bovine strain formerly described as "C. fecalis" 75205 (Al-Mashat and Taylor, 1980a and 1981) is not only similar to the other bovine and porcine CHI campylobacters but also shares common antigens with this group, confirming previous recommendations that it should be provisionally re-classified as CHI.

The absorption work and the different titres obtained by the agglutination tests have demonstrated that the surface antigens are complex in character and their reactivity is dependant on a number of antigenic factors. As was previously found in the fetus GC screening scheme the CHI antiserum NCTC11562 is specific as no positive slide agglutination was recorded with any of the standard strains or isolates of other Campylobacter spp. tested. This fact supports the provisional classification of CHI as a separate group of campylobacters which share some specific common antigens.

3) Studies on the phenomenon of black pigment of campylobacters growing on FBP containing agar

When performing Nal, TTC and C sensitivity tests on agar containing FBP it was frequently observed that the growth of CHI and to a lesser extent "C. fecalis" and C. sputorum subsp. bubulus strains produced a variable blackening. It generally occurred only in the growth area of producer strains but on occasions the blackening of some CHI strains was very strong and diffused on the agar up to adjacent strains and under antibiotic discs or TTC strips. C. jejuni, C. coli and C. fetus strains were consistently negative and no direct formation of blackening was detected. Sometimes, blackening was so strong that immediate reading of the plates was found difficult and in these cases it was noted that the pigment faded gradually after exposure of the plates to air until it completely disappeared after 3 hours. It was noted that some particular strains, as for instance CHI NCTC11562, frequently produced strong blackening but on occasions this strain and any other CHI tested were completely negative and this was related to some batches of FBP agar rather than to a difference in activity of the strains.

In order to study this phenomenon in more detail the following work was undertaken: 1) the pigment production of 25 isolates and standard strains was carefully observed after 42 hours, 3 and 4 days of incubation at  $37^{\circ}\text{C}$ -650H<sub>2</sub>; 2) the effect of FBP and sunlight exposure was studied on 7 campylobacter strains; 3) the effect of double and single components of FBP in agar after exposure to sunlight were observed with 4 standard strains.

## Materials and Methods

CHI, C. fetus, C. jejuni and C. coli isolates and standard CHI, C. fetus and "C. fecalis" strains described in Chapter 2 (Table 2.1) were used for this work.

FBP agar for tolerance tests was prepared as indicated in Chapter 2; in addition the individual constituents or combinations of the FBP chemicals were previously dissolved in water, kept in glass bottles at +4°C and added to the nutrient agar as described for the FBP compound.

Exposure of plates to sunlight was carried out immediately after pouring the plates which were left closed on the bench on a sunny day exposed to direct sunlight through the laboratory glass windows for 3 hours; this batch of medium was stored at room temperature and further exposed during storage to indirect sunlight for 1 day. Other batches of FBP agar were protected from the light as soon as the plates were poured by a cloth and when the medium had solidified, kept at +4°C in the dark until use within 24 hours of preparation.

In Experiment No. 1 a loopful of campylobacter growth was suspended in NB-FBP medium as indicated in Chapter 2. In Experiments No. 2 and 3 the same technique was followed but campylobacters were suspended in nutrient broth (without FBP) in order to avoid interference by FBP compound in the experiments.

## Results

### Experiment No. 1

Black pigment production by campylobacters on FBP agar: Nine out

of twelve CHI strains and both "C. fecalis" strains produced blackening when incubated for 42 hours at  $37^{\circ}\text{C}$ -650H<sub>2</sub>. Pigment was stronger with CHI than "C. fecalis" strains (Table 5.17A). Further incubation for 3 and 4 days generally increased the blackening which was maximum on the 3rd and 4th day of incubation. The three CHI strains which did not produce pigment remained negative up to the 4th day of incubation.

The pigmented areas were different in each test. In Nal tolerance tests an interesting phenomenon was recorded: the blackening was inhibited by the Nal activity which produced a circular white area corresponding with the diffusion of the drug from the disc. This Nal blackening inhibition zones increased their diameter when observed at the 2nd, 3rd and 4th day of incubation generally from 5 - 6 mm to 7, 8 or 9 mm. Figure 5.10A shows this phenomenon in 3 CHI (No. 5, 6 and 8) and "C. fecalis" (No. 7) strains after 4 days of incubation at  $37^{\circ}\text{C}$ -650H<sub>2</sub>; it can also be observed that overincubation reduced the described Nal sensitivity of "C. fecalis" standard strains which varied from 5 - 8 mm at 42 hours (Table 5.10A) to less than 2 mm with an irregular edge of advancing growth after the 4th day AI (Table 5.17A; Figure 5.10A, No. 7).

In TTC tolerance tests the blackening of the same 9 CHI strains and to a lesser extent the two "C. fecalis" was confined to the reddish area of growth of campylobacters without any special disposition.

In C tolerance tests the blackening of the 9 CHI strains consistently produced a 2 - 3 mm strong black area in the area of growth adjacent to the C disc and also showed a diffuse blackening usually stronger in the edges underneath the area of bacterial growth. This phenomenon was not found in "C. fecalis" strains

(Figure 5.10B, No. 5, 6 and 8).

C. jejuni, C. coli and C. fetus strains did not produce any detectable pigment on FBP agar corroborating previous observations during routine work (Table 5.17A).

Some combinations of strains showed irregular white precipitation between the areas of growth (Figures 5.10A and B). This observation could not be associated with specific strains.

### Experiment No. 2

#### Effect of FBP and sunlight exposure on the black pigment production

In this experiment the known CHI NCTC11562 strong black pigment producer strain and two CHI strains (432/83-E714 and 446/83-37) which had failed to produce blackening in the previous experiment were used (Table 5.17A). No strain produced blackening on agar without FBP either exposed or protected from sunlight (Table 5.17B). CHI NCTC11562 produced stronger blackening on FBP agar exposed to sunlight than in the non-exposed plates; CHI 446/83-37 produced strong blackening on plates exposed to sunlight and failed to produce any blackening on protected ones and CHI 432/83-E714-1 failed to produce blackening on any plate. "C. fecalis" strains produced very weak blackening on sunlight protected plates and formed notable blackening on exposed ones. C. fetus and C. jejuni did not produce blackening on any plate.

### Experiment No. 3

#### Effect of different FBP chemicals on medium pigmentation

It was found that blackening was produced in all combinations in which  $\text{Fe.SO}_4.7\text{H}_2\text{O}$  was present. No difference in the amount of

pigment produced was noted for any of the 3  $\text{FeSO}_4$  combinations tested (Table 5.17C).

### Discussion

In these experiments it was demonstrated that blackening was only produced when FBP or specifically  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  was incorporated in the agar. The  $\text{H}_2\text{S}$  production of CHI and "C. fecalis" is evidenced as blackening because  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  acts as an indicator producing black FeS (Jorgensen, 1983). The amount of blackening is not influenced by test temperatures but it is by the atmosphere used. Jorgensen (1983) demonstrated that pigment increased when hydrogen was used in the atmosphere instead of nitrogen. The importance of the atmosphere was also demonstrated in this work when it was found that after opening the jars and exposing the agar plates to air the blackening gradually diminished until complete disappearance within 3 hours, probably due to oxidation of the FeS to a colourless compound. It is interesting to compare the pigment production of these two Campylobacter spp. in Iron medium and FBP agar; "C. fecalis" strains consistently produced blackening in Iron medium but reacted weakly on FBP agar; CHI strains generally produced strong blackening on FBP agar but they were usually negative in the Iron medium. The main difference between the tests is the atmosphere of incubation as Iron medium is incubated aerobically and FBP agar at  $650\text{H}_2$ . The different pigment production of these two Campylobacter spp. in the two atmospheres may indicate distinct metabolic characters.

Nal is a specific and reversible inhibitor of bacterial DNA replication but the mechanism of action is totally unknown

(Bourguignon, Levitt and Sternglanz, 1973). At high concentrations Nal secondarily inhibits RNA and protein synthesis of Enterobacteriaceae, an action which may reduce the bactericidal activity of the compound (Crumplin and Smith, 1975). This secondary effect may explain why susceptible bacteria behave anomalously in that low concentrations of Nal are more bactericidal than high ones. It is a chelating agent (Yamabe, 1976; Timmers and Sternglanz, 1978) and its combination with  $\text{FeS}_4 \cdot 7\text{H}_2\text{O}$  blockades the latter's function as an indicator of  $\text{H}_2\text{S}$  production and is probably the cause of the Nal inhibitory effect on the blackening formation from FBP agar in tolerance tests. This same chelating property might also explain the increased Nal activity in presence of FBP compound against ovine "C. fecalis" and C. sputorum subsp. bubulus strains which are probably more sensitive than any other Campylobacter spp. to the chelated Nal compound (vide infra). In this work it was found that by overincubation "C. fecalis" strains become increasingly resistant to Nal, even in presence of FBP, showing that this effect is transient and probably reversible.

Hoffman, George, Krieg and Smibert (1979) discovered that jejuni/coli GC strains are inhibited in their growth when the agar plates were exposed to illumination by indirect sunlight. They attributed this phenomenon to the formation of photochemically generated superoxide anions and peroxide, effects which were diminished by the addition of FBP compound. They suggested that the protective action of FBP compounds was mainly due to their property of quenching toxic forms of oxygen. In this study, it was found that previous exposure of the FBP agar plates to sunlight



increased the blackening formation of CHI and "C. fecalis" strains. It is evident that FBP compounds act differently in these Campylobacter spp. than in jejuni/coli GC as these chemicals inhibit the growth of "C. fecalis" and C. sputorum subsp. bubulus. It is not known how the sunlight increased the production of FeS. It can be postulated that photo-oxidation may have induced the formation of activated oxygen which was subsequently quenched by the  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  which is thus altered to a more sensitive indicator capable of producing more FeS when incubated in a  $\text{H}_2$  rich atmosphere.

C. jejuni, C. coli and C. fetus did not produce blackening on FBP agar for tolerance tests confirming previous observations of the routine tests. This is also coincident to a negative blackening formation in Iron medium as only exceptionally C. jejuni strains were classified as biotype 2.

Differences in blackening are probably related to differences in the aerotolerance mechanism of Campylobacter spp. The microaerophilic nature of these campylobacter strains varies greatly, however one group are more "anaerobic" campylobacters in their oxygen tolerance. C. sputorum subsp. bubulus (Loesche, Gibbons and Socransky, 1965), "C. fecalis" (Firehammer, 1965) and CHI (Chapter 3, Section A) generally grow better with 3 per cent of oxygen; C. laridis generally tolerates less oxygen than jejuni/coli GC (Bolton and Coates, 1983b) and may also be included in this group. It is interesting to note that all these more "anaerobic" microaerophilic campylobacters are able to produce blackening from either Iron medium or FBP agar, whereas other more oxygen tolerant microaerophilic campylobacters such as C. jejuni biotype 1,

C. coli and C. fetus which generally require an optimum between 5 to 10 per cent of oxygen to grow (Smibert, 1978; Bolton and Coates, 1983b) usually do not produce blackening in these media.

It is worth mentioning that some campylobacters which produce black pigment from Iron medium such as C. sputorum subsp. bubulus, "C. fecalis" and C. laridis are also inhibited in their growth by FBP compound (this thesis; Bolton and Coates, 1983b) whereas strains which generally do not produce blackening from Iron medium, such as CHI, C. fetus, C. jejuni biotype 1 and C. coli are not affected or are favoured in their growth, aerotolerance and survival by FBP compound (Smibert, 1978; George, Hoffman, Smibert and Krieg, 1978; Hoffman, Krieg and Smibert, 1979; Chou, Dular and Kasatiya, 1983).

#### 4) Studies on the action of FBP components on the growth and nalidixic acid sensitivity of Campylobacter spp.

In previous works it was discovered that C. sputorum subsp. bubulus and "C. fecalis" from ovine faeces, were the only Campylobacter spp. which did not grow or grew less on standard FBP agar than in agar without FBP. Further studies on agar without FBP showed that these two Campylobacter spp. were consistently resistant to Nal and the addition of FBP to the agar was responsible for a transient Nal sensitivity. Later it was found that Nal specifically inhibited the production of blackening on FBP agar and this action was attributed to the formation of a chelated

compound between Nal and the ferrous salt of the FBP. It was then postulated that this unstable chelated compound could also be responsible for the increased Nal sensitivity of otherwise resistant ovine "C. fecalis" and C. sputorum subsp. bubulus strains.

This work was carried out with the purpose of obtaining additional information about this phenomenon. Three experiments were carried out:

Experiment A: this was performed to establish which of the three FBP components was responsible, alone or in combination with any of the others, for the depressive effect on the growth and for an increased sensitivity to Nal upon campylobacters affected by FBP compound. In this experiment ovine "C. fecalis" and C. sputorum subsp. bubulus which had and had not grown on FBP agar were selected. CHI and C. fetus subsp. fetus standard strains were used as controls.

Experiment B: a short experiment was carried out to examine the effect of the suspending fluid using the same campylobacter strains of experiment A. Campylobacters were suspended in NB-FBP or Ringer's solution and a loopful of this suspension was cultivated on 0.05 per cent ferrous sulphate 0.05 per cent sodium metabisulphite agar to determine growth.

Experiment C: this experiment was carried out to examine the effect of different concentrations of ferric and ferrous salts upon some "C. fecalis" and C. sputorum subsp. bubulus strains isolated from ovine faeces and also bull's preputial washings. The behaviour of porcine and bovine CHI, C. fetus subsp. fetus and C. jejuni strains was comparatively studied.

#### Materials and Methods

The relevant CHI, C. fetus, "C. fecalis", C. sputorum subsp. bubulus and C. jejuni biotype 2 standard strains used in the experiments are described in Table 2.1. "C. fecalis" and C. sputorum subsp. bubulus ovine isolates have been described previously (Tables 5.13 and 5.15). CHI type 1 213/83-B293-19 was isolated from the A.D.R.A. survey and used for experimental infection of lambs (Chapter 6).

Nal sensitivity test on FBP agar was carried out as described in Chapter 2. Combinations of different FBP chemicals and ferric chloride were previously dissolved in water, stored and added to the agar in the same way as was described for the FBP compound (Chapter 2). Final dilutions of  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  and  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  were prepared to contain the similar  $\text{Fe}^{++}$  and  $\text{Fe}^{+++}$  molarity respectively.

In experiment A campylobacters were suspended before cultivation in NB-FBP, in experiment C in nutrient broth (without FBP) and in experiment B in both, NB-FBP or Ringer's solution.

## Results

### Experiment A

Nalidixic acid sensitivity and growth of campylobacters on agar with and without the addition of different combinations of FBP components

C. fetus subsp. fetus and CHI standard strains grew well and were resistant to Nal in agar with or without FBP or with any of the FBP components at their standard concentration of 0.05 per cent (w/v) (Table 5.18).

"C. fecalis" and C. sputorum subsp. bubulus ovine isolates grew well and were resistant to Nal in agar without FBP compounds

or in agar containing 0.05 per cent (w/v) of either sodium pyruvate and sodium metabisulphite or only sodium pyruvate. Addition of sodium metabisulphite alone produced weak growth of all these Campylobacter spp. but sodium metabisulphite combined with ferrous sulphate inhibited completely their growth. All these campylobacters grew weakly and became sensitive to NaI or did not grow at all when complete FBP, ferrous sulphate and sodium pyruvate or ferrous sulphate alone were added to the agar (Table 5.18).

#### Experiment B

##### Growth of campylobacters on ferrous sulphate-sodium metabisulphite agar

CHI and C. fetus subsp. fetus standard strains grew well on 0.05 per cent of ferrous sulphate-sodium metabisulphite agar either previously suspended in NB-FBP or Ringer's solution.

"C. fecalis" and C. sputorum subsp. bubulus only grew on ferrous sulphate-sodium metabisulphite agar when previously suspended in Ringer's solution, but failed to grow when NB-FBP was used.

#### Experiment C

##### Nalidixic acid sensitivity and growth of campylobacters on agar with and without the addition of different concentrations of ferrous and ferric salts

The action of ferrous sulphate (Table 5.19A) and ferric chloride (Table 5.19B) on campylobacters was very similar. All campylobacters grew well on agar without added ferrous or ferric salts. In the same media C. jejuni biotype 2, three C. sputorum subsp. bubulus and one "C. fecalis" strain isolated from bull's preputial washings

were sensitive to Nal; the rest of the tested strains were resistant to Nal in absence of iron salts.

C. jejuni biotype 2 grew well up to concentrations of 5.4 mM of ferric or ferrous salts but it did not grow at levels of 7.2 mM. It was sensitive to Nal at all concentrations in which growth was recorded but the Nal inhibition haloes of growth became narrower as the iron concentration increased.

CHI and C. fetus subsp. fetus strains grew well up to 3.6 mM of iron salts, most strains grew weakly at 5.4 mM and they generally did not grow at 7.2 mM, except for C. fetus subsp. fetus and the bovine CHI 213/82-B293-19 which grew weakly at 7.2 mM of  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ . These strains were consistently resistant to Nal even at the iron concentrations in which weak growth was recorded.

"C. fecalis" and C. sputorum subsp. bubulus were all more affected in their growth by the iron concentrations than any of the other Campylobacter spp. No strain grew at 5.4 mM of iron salts and weak growth was recorded in some strains from 0.9 mM onwards. All strains which were resistant to Nal in medium without added iron salts became sensitive to Nal in presence of them; all but C. sputorum subsp. bubulus NCTC10355 became sensitive from 0.9 mM onwards and this last strain became sensitive to Nal from 1.8 mM onwards.

### Discussion

In experiment A (Table 5.18) it was demonstrated that ferrous sulphate and sodium metabisulphite have both an inhibitory effect on the growth of "C. fecalis" and C. sputorum subsp. bubulus whereas CHI type 1 and C. fetus subsp. fetus strains are not affected.

Sodium pyruvate alone did not affect the growth of any strain and diminished the inhibitory growth effect of sodium metabisulphite. In fact, on agar with only sodium metabisulphite added all the affected campylobacter group strains grew weakly whereas these bacteria grew well when sodium metabisulphite was combined with sodium pyruvate. On the other hand, sodium pyruvate did not reduce the inhibitory effect of ferrous sulphate. Hence maximum inhibitory effect was produced when ferrous sulphate and sodium metabisulphite were combined without added sodium pyruvate, a combination in which no "C. fecalis" or C. sputorum subsp. bubulus strains grew. It is worth mentioning that Jorgensen (1983) found that sodium metabisulphite hastened the development of blackening from ferrous sulphate by "thermophilic campylobacters" (jejuni/coli GC and C. laridis, HT), probably another expression of the synergistic effect of the chemicals.

In experiment B it was corroborated that CHI and C. fetus subsp. fetus were the only strains tested able to grow on ferrous sulphate-sodium metabisulphite when the inoculum was suspended in NB-FBP; in contrast, none of the "C. fecalis" or C. sputorum subsp. bubulus strains grew. On the other hand, when the campylobacters were suspended in Ringer's solution "C. fecalis" and C. sputorum subsp. bubulus strains grew in this medium. Probably, a synergistic or simple additive effect of both the FBP components of the broth and the two chemicals of the agar are responsible for the inhibition of growth. Although in this work no further studies were carried out it could be suggested that a standardized ferrous sulphate-sodium metabisulphite test might prove useful



in taxonomic studies to separate organisms of the fecalis and subsp. bubulus groups.

Although 0.025 (George, Hoffman, Smibert and Krieg, 1978) and 0.05 per cent (Hoffman, George, Krieg and Smibert, 1979) of sodium metabisulphite improved the aerotolerance and growth of jejuni/coli GC when used as part of the FBP compound, recently Koidis and Doyle (1983) found that sodium bisulphite alone increased the survival of C. jejuni at levels of 0.01 per cent but at levels of 0.05 per cent it was toxic for this Campylobacter sp. which died more rapidly in medium containing this level of bisulphite than in medium free of added bisulphite. The results reported here corroborate the findings of this work in which it was demonstrated that sodium pyruvate from FBP diminished the toxicity of sodium metabisulphite.

When in experiment C ferrous and ferric salts were comparatively studied at different concentrations it was found that both similarly inhibited the growth of campylobacters. Both salts proved more inhibitory to the growth of all "C. fecalis" and C. sputorum subsp. bubulus strains tested than the C. jejuni, CHI or C. fetus subsp. fetus. George, Hoffman, Smibert and Krieg (1978) found that C. sputorum subsp. bubulus strains grew on both brucella agar with and without FBP and colony counts were similar in both media. In their experiments they used 0.025 per cent of each FBP component and described that the oxygen tolerance of C. sputorum subsp. bubulus strains appeared not to be increased by FBP. In experiment C similar concentrations of 0.024 and 0.025 per cent of ferric and ferrous salts were respectively used

(without any other added FBP component) and it was found that all "C. fecalis" and C. sputorum subsp. bubulus strains grew although a few did so weakly. In this experiment colony counts were not done so it is not possible to know if at this concentration weak growth represents fewer colonies of campylobacters or only similar numbers of smaller colonies.

Bolton and Coates (1983a) found that counts of organisms on basal medium containing ferrous sulphate or ferric nitrate increased with iron concentration until the level became inhibitory; 0.1 per cent inhibited the growth of C. lariidis and 0.2 per cent inhibited the growth of C. jejuni and C. coli standard strains. These results are in agreement with those of experiment C in which C. jejuni biotype 2 did not grow at levels of 0.2 and 0.19 per cent of ferrous sulphate and ferric chloride respectively (Tables 5.19).

In experiment A (Table 5.18) it was demonstrated that ferrous sulphate was the only FBP component responsible for the Nal sensitivity of "C. fecalis" and C. sputorum subsp. bubulus faecal ovine strains, as these campylobacters were resistant to Nal in agar containing sodium metabisulphite and/or sodium pyruvate. In experiment C (Table 5.19) it was found that "C. fecalis" and C. sputorum subsp. bubulus (isolated from the bovine genital tract) were sensitive to Nal in agar without FBP except for C. sputorum subsp. bubulus NCTC10355 strain. At levels of 0.024 and 0.025 per cent of ferric or ferrous salts respectively, all the "C. fecalis" and C. sputorum subsp. bubulus strains grew and were sensitive to Nal. In general, it was observed that "C. fecalis"

and C. sputorum subsp. bubulus strains enlarged the size of their Nal inhibition zones when the iron concentration increased but interestingly in C. jejuni biotype 2 standard strain a reverse phenomenon was found in both iron salts tested.

Harvey and Greenwood (1983) described that ovine "C. fecalis" strains had variable susceptibility to Nal acid. They studied five strains received from Firehammer different from those used in this work. In their experiments all the ovine "C. fecalis" and only one of the 5 bovine C. sputorum subsp. bubulus strains examined were resistant to Nal when agar without FBP was used. As in experiment C nutrient broth without FBP was employed, it has to be concluded that these 4 bovine strains were Nal sensitive in absence of added iron. The variability of Nal resistance in this group of campylobacters may be due to the presence of transmissible R factors. These factors are widely known for their ability to increase the resistance of Gram negative bacteria to the effect of antimicrobial agents but their presence may also make bacteria more susceptible to the inhibitory effects of certain antibacterial agents. Several unrelated R factors increased the susceptibility of their bacterial host strain to the bactericidal effect of Nal and this increased susceptibility results from an increase in the permeability of the cell wall facilitating an increase uptake of the drug (Crumplin and Smith, 1981).

Yamabe (1976) demonstrated that Nal increased rate of electron transfer from ferrous sulphate to cytochrome c in in vitro experiments. This investigator suggested that the model may represent the actual bacterial electron transfer system and postulated that

Nal is able to chelate  $\text{Fe}^{++}$  and such chelated compounds have affinity for the cytochrome c molecule probably forming a multi-component complex as  $\text{Fe}^{++}$ -Nal-cytochrome. Then certain electron transfer system closely linked with bacterial DNA synthesis is abnormally accelerated, resulting in an interference with nucleus formation. Other studies on Nal indicate that this drug may act by forming in situ a complex with a divalent cation in a metalloprotein involved in bacterial DNA replication (Timmers and Sternglanz, 1978). In these experiments both ferrous and ferric salts acted similarly but this may well be due to previous reduction of  $\text{Fe}^{+++}$  to  $\text{Fe}^{++}$  by the campylobacters. The fact that only one group of campylobacters is affected by iron salts probably reflects the presence of different electron transfer systems and perhaps the presence of different cytochromes in "C. fecalis" and C. sputorum subsp. bubulus from CHI and C. fetus.

5) Campylobacter anaerobic growth in presence of nitrate, aspartate and fumarate

Razi, Park and Skirrow (1981) found that generally fetus GC strains were able to grow in presence of either nitrate or L-aspartate whereas jejuni/coli GC strains failed to do so. They also suggested that anaerobic growth in the presence of fumarate might be useful for finer differentiation between C. fetus strains.

The purpose of this work was to ascertain if these tests could be used to differentiate fetus GC.

### Materials and Methods

CHI, C. fetus, "C. fecalis" and C. sputorum subsp. bubulus isolates and standard strains used have been described in Tables 2.1 and 5.13. C. jejuni biotype 2 NCTC11392 was used as negative control for the tests.

YNB supplemented with potassium nitrate, potassium L-aspartate, sodium fumarate and unsupplemented medium were prepared as described in Chapter 2. Two tests were carried out: 1) test tubes incubated at 37°C-ANA-O<sub>2</sub>-plus and 2) McCartney bottles incubated at 37°C-O<sub>2</sub> with lids tightly screwed down. These tests were performed and interpreted as described in Chapter 2.

### Results and Discussion

The test using bottles was easy to perform and more strains could be investigated without need for anaerobic jars but in many cases it was found that doubtful results occurred in which surface or immediate sub-surface growth was heavy but also weak growth was detected in the media; these doubtful results were recorded as negative if they did not develop further after the third reading at 7 days of incubation.

The test in tubes incubated anaerobically was found to be more reliable and a sharp difference between positive and negative results was recorded.

C. jejuni biotype 2 NCTC11392 did not grow either in tubes or bottles of supplemented and unsupplemented media confirming previous observations (Tables 5.20A and B). On the other hand, all fetus GC strains grew in at least one of the supplemented media and failed to grow in unsupplemented medium.

All CHI strains grew in nitrate medium either in tests using tubes or bottles but many aspartate and fumarate tests were negative. C. fetus strains except the standard NCTC5058 grew in nitrate, whilst all these strains grew in aspartate and fumarate; C. fetus 311/83-10 did not grow in aspartate in the anaerobic test in tubes but grew in the bottle test. Razi, Park and Skirrow (1981) found similar results as most of C. fetus strains grew in nitrate and aspartate but a few failed to grow in aspartate or both media. Most "C. fecalis" grew in nitrate, aspartate and fumarate but one failed to grow in nitrate and another in both aspartate and fumarate (Tables 5.20A and B).

The tests using tubes incubated at 37°C-ANA-O<sub>2</sub>-plus could be useful as a complementary test to differentiate most of jejuni/coli GC strains from fetus GC but in this study they were not found useful to classify members of the fetus GC. Véron, Lenvoisé-Furet and Beaune (1981) noted that the anaerobic fumarate respiration in the genus Campylobacter has taxonomic implications as this test differentiates C. jejuni (non-grower) from C. fetus subsp. fetus (grower). This work corroborates these findings as all C. fetus tested grew in both tests in fumarate supplemented YNB medium whereas C. jejuni did not.

In general CHI strains appeared to differ from C. fetus in that the latter grew with aspartate (oxygen tube test), however all strains did not conform to a pattern and in general the difficulty of reading the test clearly limits its usefulness.

#### 6) Detection of capsule in Campylobacter spp.

Pathogenic bacteria survive in host tissues due to their ability to resist phagocytosis. A superficial antigen of C. fetus subsp. fetus named "antigen a" comprises the microcapsule and is a glycoprotein. Cells possessing this antigen are refractory to ingestion by macrophages except in the presence of specific antiserum but in its absence maximum phagocytosis occurs. Its principal biological function is as antiphagocytic effect and there is also evidence that it protects the bacterium from antibody-mediated immobilization (McCoy, Doyle, Burda, Corbeil and Winter, 1975; McCoy, Wiltberger and Winter, 1976; Winter, McCoy, Fullmer, Burda and Bier, 1978).

Due to the importance of the capsule in the pathogenesis of other campylobacter diseases its presence was sought by staining techniques in some campylobacter isolates, standard strains and campylobacters re-isolated from the studies of experimental production of disease in gnotobiotic calves and lambs.

#### Materials and Methods

Twenty four C. jejuni, 3 C. coli, 9 CHI type 1, 3 CHI type 2, 7 C. fetus, 6 "C. fecalis" and 2 C. sputorum subsp. bubulus strains were used for the present study. Ten C. jejuni biotype 1, one C. coli and one CHI type 1 were strains re-isolated from gnotobiotic calves and lambs; information about the animal, origin and day PI when the strains were isolated are shown in Table 5.21A. Eight strains were used to compare the presence of capsule in M and N type of C. jejuni (vide infra, Chapter 6). The rest of



the strains were isolated from A.D.R.A., control farms, diagnostic material, slaughterhouse and Easter Bush surveys or were standard strains (Table 5.21B).

A loopful from CBA growth incubated at  $37^{\circ}\text{C}$ -650H<sub>2</sub> for 2 days was suspended in NB-FBP medium and smears were prepared from a drop of this suspension according to the Benian's Congo red technique described by Chatterjee and Neogy (1972).

### Results

The capsule of campylobacters was evident around the cells as a fairly thick and unstained halo corresponding to the length of the bacillus or coccal body (Figures 5.11). The capsule was unstained but the external surface was distinguishable due to a stronger red colour than the faint reddish-violet background. The central part of the halo was occupied by the body of the organism which was stained a deep violet tint. Sometimes a weak, less well defined capsule was noted. Campylobacters and coccal forms without capsule were often seen together with strongly or weakly capsulated campylobacters. Only two C. jejuni strains were found to be homogeneous acapsulated bacterial strains (Table 5.21B).

In general, all Campylobacter spp. have capsules but it was more common to find well formed homogeneous capsulated campylobacters among the fetus GC members examined than in jejuni/coli GC strains (Tables 5.21A and B).

C. jejuni biotype 1 type N colonies isolated from the colon of a gnotobiotic calf at 14th day PI yielded campylobacters and coccal forms with well formed capsules whereas M type colonies

isolated from the same animal at the same time showed most bacteria and coccal bodies without capsules. Nevertheless, examination of 2 other groups of M and N type colonies simultaneously isolated from the same animal and from another calf showed no differences between the types (Table 5.21A).

### Discussion

The findings of this work revealed that campylobacters of all species generally have a well formed and defined capsule. It was common to observe occasional capsulated bacteria among a majority of non-capsulated cells and also when the same strain was re-isolated from the same animal on occasion the culture was predominantly capsulated and at other times non-capsulated. This variability indicates that capsulated/non-capsulated variation may be common among campylobacters and not a fixed characteristic of a strain. As all cultures examined for capsular staining were grown on the same medium and identical incubation procedures were followed it is unlikely that these variations were caused by the bacteriological techniques used.

It is interesting to note that frequently when coccal forms were found in a capsulated strain they also displayed a notable and very well defined capsule (Figure 5.11B). Most authors believe that coccal forms are degenerative stages of campylobacters, but nevertheless the presence of this well formed capsule in some coccal forms of young cultures not exposed to oxygen may indicate that at least some of these spheres could be expression of a life cycle. Hallock (1959) studied the different coccal forms of Vibrio spp. and concluded that some of the spheres were in fact

resting cells which revert to vibrio cells when active multiplication takes place. Similar studies have not been performed on Campylobacter spp.

In the work of this thesis young cultures of the C. jejuni biotype 1 1644/82-D523-2-CO type N consistently formed numerous capsulated coccal forms when incubated for only 18 hours at 43°C-550H<sub>2</sub> or 42 hours at 37°C-650H<sub>2</sub> whereas the M type produced fewer coccal forms at the same period. Subcultures of this predominantly coccoid N type culture consistently produced abundant growth on CBA plates. Further studies of these strains will be referred to in next chapter.

It is not known if the same "antigen a" which comprises the microcapsule of some C. fetus subsp. fetus strains is in fact the same capsule detected by the staining technique. It proved possible to isolate a capsulated wild-type strain and a non-capsulated mutant from the same strain (McCoy, Doyle, Burda, Corbeil and Winter, 1975) these differed in being stable variants.

Capsular antigens of C. jejuni have not been studied in so much detail as those of C. fetus but Merrell, Walker and Coolbaugh (1982) identified a capsule-like material on the surface of C. jejuni by ruthenium red staining.

CHAPTER 6CAMPYLOBACTER EXPERIMENTAL INFECTIONS IN GNOTOBIOTIC RUMINANTSIntroduction1) Campylobacter enteric infections

Jejuni/coli GC have been recently recognized as a cause of diarrhoea in man. In contrast, campylobacters have been demonstrated in calves faeces more than half a century ago (Smith and Orcutt, 1927) but their role as etiological agents has not been convincingly demonstrated. Early research suggested that they might be a cause of calf scour (Jones and Little, 1931b) but these studies were carried out when the other agents of calf diarrhoea were unknown and the disease was produced in most cases by feeding calves with faeces obtained from spontaneous cases of disease (Jones, Little and Orcutt, 1932; Jones, 1933). Al-Mashat and Taylor (1980b) produced a mild abomasitis and ileitis in conventional ruminating and milk-fed calves with cultures of jejuni/coli GC. Firehammer and Myers (1981) produced diarrhoea in 3 out of 12 conventional young calves orally inoculated with jejuni/coli GC, but 2 of them were known to be compromised by other disorders at the time of challenge exposure. The results and conclusions of all these experiments are limited by the almost inevitable presence of intercurrent bacterial and viral pathogens. Recently, Morgan, Hall, Reynolds and Parsons (1983) orally infected five gnotobiotic calves with mixtures of C. jejuni and C. coli and produced a mild illness characterised by fever and excretion of mucoid soft faeces.

It has been demonstrated in Chapter 5 that the described porcine C. coli type I (Lawson, Rowland and Wooding, 1975) and CHI (Gebhart, Ward, Chang and Kurtz, 1983) strains and a bovine "C. fecalis" strain (Al-Mashat and Taylor, 1981) were biochemically similar and serologically

related to a group of bovine campylobacters frequently isolated from the bovine surveys of this thesis and provisionally classified as CHI type 1. Al-Mashat and Taylor (1981) and Taylor and Al-Mashat (1984) described the production of abomasitis and ileitis in six milk-fed and ruminant conventional calves by oral inoculation of "C. fecalis", the strain re-classified in this thesis as CHI type 1 (Al-Mashat, Personal Communication, 1983). Porcine CHI strains have been detected by an immunoperoxidase test within enterocytes of ileal crypts in natural cases of porcine intestinal adenomatosis and it was suggested that this campylobacter might be involved in this disease (Chang, Ward, Gebhart and Kurtz, 1983). Intraepithelial campylobacter-like organisms have been found in cases of regional ileitis of lambs, another proliferative enteropathy occasionally recorded (Hoorens, Oyaert, Meyvisch, Vandenberghe and Derijcke, 1977). Preliminary cultural work has recovered both campylobacters resembling C. sputorum and C. jejuni although it is not known if these microorganisms are or are not the intracellular vibrioid bacteria (Vandenberghe and Hoorens, 1980).

Allsup, Matthews, Hogg and Hunter (1972) produced intermittent scouring in two conventional milk-fed calves by oral inoculation of Campylobacter spp. (probably C. fetus subsp. fetus, HT). Later, Al-Mashat and Taylor (1983b) fed six milk-fed and ruminant conventional calves with cultures of C. fetus subsp. fetus and produced ileitis and to a lesser extent, jejunitis and abomasitis. In these experiments other potential enteropathogens were detected which may have distorted the pathological findings.

Russell (1955) observed numerous vibrios in two cases of profuse watery scour in hoggets and suggested that campylobacters might be the

cause of the disease. Later on, Bryans and Shephard (1961) orally infected conventional lambs with cultures of jejuni/coli GC and detected watery diarrhoea in 3 out of 11 infected animals. Firehammer and Myers (1981) orally inoculated 5 conventional lambs with jejuni/coli GC cultures and although none of the animals developed diarrhoea the faeces became mucoid and intermittently contained flecks of blood.

The few experimental oral infections of sheep with C. fetus subsp. fetus were to examine excretion and the effects of infection upon the reproductive tract and enteric changes were not described (Taylor and Al-Mashat, 1984).

Recently, Roberts (1983) infected both sheep and cattle with a wide range of Campylobacter spp. from human, animal and avian sources. These animals were reared campylobacter free. The only strain which did not colonize after oral inoculation was C. laridis. Infected animals remained clinically healthy and passed variable amounts of mucus in faeces, although stool consistency was usually not altered. Reinfection experiments demonstrated that resistance to homologous but not heterologous challenge occurred.

"C. fecalis" was isolated from the faeces of normal sheep and was considered to be a normal inhabitant of the ovine gastrointestinal tract (Firehammer, 1965; Smibert, 1978). There are no reports of experimental infections of sheep or cattle with this organism.

## 2) Mechanisms of pathogenicity

### 2.1 Attachment

The attachment of pathogenic organisms to epithelial cells is a necessary pre-requisite to invasion of the intestinal mucosal surface. Attachment of campylobacters has been studied in vitro by means of

tissue cultures, isolated brushborders and cells in suspension.

Dijs and de Graaf (1982) found that 10 C. jejuni strains tested failed to show adhesion to epithelial cells of brushborders isolated from the jejunum of piglets and observed by phase-contrast microscopy. However, Naess, Johannesssen and Hofstad (1983) observed by SEM that C. jejuni strains NCTC11168, NCTC11392 and CTC11351 adhered, probably non-specifically, to both the porcine brushborder and the adjacent enterocyte cell fragments and similarly Al-Mashat (1981) and Taylor and Al-Mashat (1984) noted an association between C. jejuni, C. fetus subsp. fetus and CHI strains and brushborders isolated from the intestine of calves which was markedly looser than that of E. coli control preparations.

Most strains of C. jejuni showed poor spontaneous adhesion to epithelial cell lines of Hela or INT 407 (Manninen, Prescott and Dohoo, 1982; Newell and Pearson, 1982) although C. coli may differ. Others more recently (Cinco, Banfi, Ruaro, Crevatin and Crotti, 1984) have found that all eleven C. jejuni and three C. coli strains examined showed rapid adhesion to INT 407 cells within 30 minutes, eluting thereafter.

McBride and Newell (1983) found that attachment involves a close association between the bipolar flagella and the cells surface. These authors compared the attachment of a wild flagellate/motile C. jejuni strain with two selected variants, and found that an aflagellate/non-motile variant attached relatively poorly to epithelial cell lines monolayers whilst a flagellate/non-motile variant attaches more successfully than the wild flagellate/motile strain.

In contrast with these previous results the same authors found that aflagellate variants attached significantly better than the wild type



or flagellate/non-motile variants to suspensions of red blood cells from various animal species or human buccal cells. Probably, a second adhesin present on the surface of C. jejuni is responsible for this attachment and active flagellae and to a lesser extent inactive flagellae hinder the interaction between this second adhesin and the target cell. Haemagglutination of erythrocytes of various species was shown to be associated with several different adhesins in the cell wall of jejuni/coli GC (Lastovica, 1983) and different strains displayed distinct haemagglutination activity (Cinco, Banfi and Crotti, 1983). Merrell, Walker and Coolbaugh (1982) suggested that attachment of campylobacters to intestinal epithelial cells may be mediated by a capsule possessed by C. jejuni and identified by EM using ruthenium red staining. Fimbriae or pili were not observed on jejuni/coli GC (Dijs and de Graaf, 1982; Lastovica, 1983).

McCardell, Madden, Bier, Lee and Dallas (1983) demonstrated in vivo the adhesion of C. jejuni to villi in rabbit ligated ileal loops. Interestingly, adhesion of bacteria to villi only occurred in a few areas in which mucus was absent. Fauchere, Véron, Pfister and Lellouch-Tubiana (1983) observed by EM bacterial adherence of C. jejuni on the intestinal mucosa of adult germ-free or monoxenic mice.

## 2.2 Colonization

The most important features of campylobacter colonization can be summarized as follows:

- a) Successful colonization: In most experiments colonization readily takes place and vast numbers of campylobacters can be recovered (Newell, 1984).
- b) Interference of normal flora: When gnotobiotic chickens or mice are

previously colonized by normal flora subsequent campylobacter colonization is largely prevented. Furthermore, oral inoculation of normal flora to campylobacter colonized chickens or mice provokes elimination of campylobacters (Soerjadi, Snoeyenbos and Weinack, 1982 and 1984; Andreumont, Leonard, Goldstein, Pean, Pequet and Tancrede, 1983). In gnotobiotic pigs the presence of selected enteric bacteria might have increased the total numbers of C. sputorum subsp. mucosalis whereas multiple undefined bacterial contamination appears to depress the numbers of campylobacters (McCartney, Lawson and Rowland, 1984).

c) Difference in colonization between gnotobiotic or neonatal and conventional animals: When jejuni/coli GC was inoculated to gnotobiotic calves (Morgan, Hall, Reynolds and Parsons, 1983), piglets (Andress, Barnum and Thomson, 1968; Kashiwazaki, Namioka and Yabiki, 1971) and dogs (Prescott, Barker, Manninen and Miniats, 1981), and neonatal mice, rats and rabbits (Field, Underwood, Pope and Berry, 1981) higher numbers of the bacteria were detected in the caecum and large intestine than in the small bowel. Inversely, if adult conventional mice were infected the numbers of C. jejuni were higher in the small intestine than in the caecum and colon (Blaser, Duncan, Warren and Wang, 1983).

d) Localization of campylobacters in the intestine: In conventional calves (Taylor and Al-Mashat, 1984), infant mice (Field, Underwood, Pope and Berry, 1981), gnotobiotic dogs (Prescott, Barker, Manninen and Miniats, 1981) and chickens (Ruiz-Palacios, Escamilla and Torres, 1981) organisms with campylobacter morphology were observed adjacent to the mucosal epithelium and in the crypts of the intestine by light and electron microscopic techniques. In mice and rabbits numerous organisms were visible on, in and below dried mucus layers observed by

SEM (Field, Underwood, Pope and Berry, 1981; McCardell, Madden, Bier, Lee and Dallas, 1983).

e) Different colonization of strains of the same Campylobacter spp.:

Different jejuni/coli GC strains have different ability to colonize and persist in the same host (Field, Underwood, Pope and Berry, 1981).

f) Presence of flagella and colonization: Flagellate/motile wild C. jejuni strains and a flagellate/non-motile variant colonized the gut of infant mice and could be recovered from the colon, caecum, small intestine and faeces for up to 30 days PI. In contrast, an aflagellate/non-motile variant colonized the intestine poorly and few, if any, microorganisms could be recovered after the 7th day PI (Newell, McBride and Dolby, 1983).

g) Campylobacters as specific mucosa-associated microorganisms: In mice oxygen-intolerant fusiform shaped bacteria and spiral shaped microorganisms lie in intimate contact with the mucin layer and in close proximity to discharging goblet cells in the crypts of caecum and colon (Savage, McAllister and Davis, 1971). Similar microorganisms have been described in dogs and pigs and recently Lee, O'Rourke, Phillips and Barrington (1983) proposed that C. jejuni is a mucosa-associated bacterium specifically adapted to colonize the mucus environment of the intestinal surface. They compared the colonization of C. jejuni and a microaerophilic spiral organism isolated from the rodent gut and found that both had the same preferential sites of colonization of gnotobiotic mice: many caecal crypts were colonized by large numbers of bacteria whereas few crypts of the ileum and colon were colonized by small numbers. Due to the characteristic morphology of the spiral microorganism it was possible to recognize it from other bacteria in

conventional mice, where it was found to preferentially colonize the small intestine rather than the large bowel. This observation is important and explains to some extent the different results obtained by other investigators working with jejuni/coli GC in gnotobiotic, neonatal or conventional animals.

The same authors found that both C. jejuni and the spiral bacteria were better able to move in solutions with a viscosity approximating intestinal mucus and in this they differed from other gut organisms. This property is an ecological advantage in the mucus-filled intestinal crypts. These investigators suggested that colonization of mucus is an essential step in the pathogenesis of campylobacter infection.

Similar studies on fetus GC in the gastrointestinal tract have not been carried out but investigations with C. fetus subsp. isolated from the bovine and ovine genital tract indicate that fetus GC are well adapted to persist in the mucilaginous environment. Furthermore, C. fetus subsp. are able to produce mucinase which causes depolymerization of glandular mucins and resembles the mucinolytic activity of Vibrio spp. (Dennis, 1967b).

### 2.3 Invasion

Some experimental infections suggest that invasion might be a mechanism of campylobacter diarrhoea. Ruiz-Palacios, Escamilla and Torres (1981) found that after oral infection of 3-day-old chickens jejuni/coli GC replicated in the small intestine, 12 hours later it was seen by EM penetrating the intestinal epithelium of the jejunum and ileum and in 24 hours using immunofluorescence techniques antigen was visualized within phagocytic cells in the lamina propria. Similar findings were reported by Butzler and Skirrow (1979). Nevertheless,

in other experiments with other species invasive ability of C. jejuni was not demonstrated. Prescott, Barker, Manninen and Miniats (1981) found that campylobacter established at over  $10^{10}$  organisms per gram of colonic content of gnotobiotic dogs but did not invade the mucosa; these authors observed at the ultrastructural level organisms in close proximity to the mucosa and suggested that C. jejuni was probably capable of inducing pathological changes by releasing toxic factors.

Taylor and Al-Mashat (1984) found that when calves were killed at 24 hours PI C. jejuni was recovered from the MLN, gallbladder, lung and spleen, but at 48 hours PI campylobacters were only recovered from the gastrointestinal tract, MLN and spleen. By 96 hours PI C. jejuni could only be recovered from the ileum, caecum and colon. These findings may be indicative of a transient bacteremia at the beginning of the infection. Transient bacteremia by C. jejuni was demonstrated within 10 minutes of infection and not for more than 3 hours in both orally inoculated mice (Blaser, Duncan, Warren and Wang, 1983) and intra-ileum inoculated rabbits (McCardell, Madden, Bier, Lee and Dallas, 1983). Different results were obtained by Bryner and Warner (1983) who recovered C. jejuni and C. coli from the blood of orally inoculated calves up to 42 days PI. This notable difference could be due to the strains used, in the latter experiment the C. jejuni and C. coli strains had been isolated from an ovine and a swine aborted fetus respectively, whereas the strains employed in the other experiments were from the intestinal tract.

Kita, Katsui, Yanagase and Kashiba (1983) found that only 10 out of 96 human C. jejuni strains, isolated from cases of diarrhoea, had ability to invade the epithelium of mouse uterus. The most invasive strain had

significant amounts of high molecular weight heat labile membrane proteins.

The penetration of chicken embryonic chorioallantoic membrane by human isolates of C. jejuni provides further evidence for an invasive capacity. Interestingly, two strains tested displayed differences in their ability to invade and the authors suggested that inoculating 11-day-old chicken embryos and examining them at 48 hours could be used as a possible model to compare the invasive ability of different strains (Davison and Solomon, 1981).

Cell cultures have been used to study the invasive ability of campylobacters as well as adherence which has been described earlier. Penetration involves a progressive movement of the organism into the cell from a polar position. Although several campylobacters may be found in a vacuole there is no direct evidence of intracellular replication and the rapid cell death probably occurs by a cytotoxic phenomenon (Newell, 1984). C. sputorum subsp. mucosalis associated with the production of proliferative enteritis in pigs have the ability of not only invading some specific culture cells but also of multiplying intracellularly whereas CHI induces rapid cell death (Rajasekhar, 1981).

The Séreny Guinea-pig conjunctival sac test was consistently negative for C. jejuni and C. coli strains (Manninen, Prescott and Dohoo, 1982; Gubina, Zajc-Satler, Dragas, Zeleznik and Mehle, 1982).

## 2.4 Toxin production

### 2.4.1 Endotoxins

In common with other Gram negative bacteria C. fetus possesses a three layer wall which consists of an outer lipoprotein layer, a middle lipo-polysaccharide layer and an inner mucopeptide layer (Keeler,

Ritchie, Bryner and Elmore, 1966). Heat-killed C. jejuni biotypes 1 and 2, C. coli and C. sputorum subsp. bubulus have endotoxin activity detected by limulus assay and dermal Schwartzman reaction in rabbits. The suspensions presented a limulus activity equivalent to 16-32 µg/ml endotoxin compared with 128 µg/ml of the E. coli cultures used as controls (Fumarola, Miragliotta and Jirillo, 1982). The significance of endotoxin in the pathogenesis of campylobacter infections has yet to be demonstrated but the inflammation and thrombosis frequently described in C. fetus infections may be the result of local endotoxin activity (Newell, 1984).

#### 2.4.2 Enterotoxins and cytotoxins

Gubina, Zajc-Satler, Dragas, Zeleznik and Mehle (1982) have described a heat labile (LT) enterotoxin which produced cell rounding (cytotoxic effect) in cultures of mouse adrenal tumour cells (Y-1) in both C. fetus subsp. fetus and C. jejuni strains. Ruiz-Palacios, Torres, Torres, Escamilla, Ruiz-Palacios and Tamayo (1983) reported the production of a different enterotoxin which induced elongation (cytotonic effect) and increased intracellular cyclic AMP levels in Chinese hamster ovary (CHO) cells. The similarities between this and cholera toxin are very interesting: both are heat labile and both raise intracellular cyclic AMP levels, probably by the same mechanism; furthermore, antibodies against whole cholera toxin block C. jejuni toxin. Despite the immunological affinity recent comparative studies on DNA hybridization demonstrated that the gene coding for enterotoxin production in C. jejuni is not closely related to the gene coding for either cholera toxin or E. coli LT (Olsvik, Wachsmuth, Morris and Feeley, 1984). This cholera-like-toxin induced fluid secretion in rat but not in rabbit ileal loop or the infant mouse assay.



McCardell, Madden and Lee (1983) confirmed that cholera-like-toxin containing cell-free supernatants were negative in the Y-1 cell cytotoxic assay but protein from ammonium sulphate-treated supernatants caused cell rounding. Johnson and Lior (1984) found that both cytotoxic and cytotoxic factors were produced by 60 and 64 per cent of C. jejuni and C. coli isolates whereas only cytotoxic factors were detected in 25 and 21 per cent of C. jejuni and C. coli isolates respectively. Infant mouse assay of the cytotoxic cytotoxic preparation were negative confirming previous works which examined ruminant and strains of other origin (Firehammer and Myers, 1981; Manninen, Prescott and Dohoo, 1982). The effect of these preparations on the ileal loop of different animal species have still to be determined but earlier work failed to detect the presence of heat stable (ST) toxin in intestinal loops of lambs, calves or pigs (Firehammer and Myers, 1981; Manninen, Prescott and Dohoo, 1982).

### 3) Pathology

Jones (1933) described that the pathology of the disease in cattle in both natural and experimental cases was consistently localized in the jejunum; the wall was edematous, the mucosa swollen, moderately reddened and overlain with mucus.

Al-Mashat and Taylor (1980b, 1981 and 1983) and Al-Mashat (1981) performed experiments in conventional calves with jejuni/coli GC, C. fetus subsp. fetus and CHI strains reporting a complete pathological description. The three Campylobacter spp. studied produced very similar lesions mainly restricted to the small intestine and characterised by stunted villi or villous atrophy, dilated crypts which sometimes contained inflammatory cells, dilated capillaries, loss of the luminal epithelium and in many cases the epithelium

of the crypt was cuboidal. Campylobacter-like organisms were found in the crypts of the caecum. There was degeneration of cells lining the large intestinal crypts and goblet cell discharge was prominent. In some animals abomasitis was reported. BY TEM shortened microvilli and damaged mitochondria were found. Bacteria with the dimensions of campylobacters were present in the cytoplasm of some of the neutrophils of the affected crypts. No bacteria could be seen within enterocytes or closely adjacent to their luminal surfaces.

In general, the lesions were present in areas in which campylobacters colonize more efficiently, and differences in distribution of intestinal lesions in gnotobiotic and conventional animals is in accordance with the distribution of the campylobacters. For instance, when conventional dogs were inoculated with C. jejuni the lesions were restricted to the jejunum and ileum (Macartney, McCandlish, Al-Mashat and Taylor, 1982) whereas when the same species was inoculated into gnotobiotic dogs significant microscopic lesions were limited to the caecum and colon (Prescott, Barker, Manninen and Miniats, 1981). Similarly, when gnotobiotic piglets were inoculated with C. coli the lesions were chiefly present in the large intestine whereas in conventional pigs the changes were in the jejunum and ileum and to a lesser extent in the colon (Andress, Barnum and Thompson, 1968; Olubunmi and Taylor, 1982).

Early lesions of the large bowel of gnotobiotic dogs tend to be patchy in distribution and consisted in cuboidal or low columnar epithelium with microerosions, mild edema and inflammation of the lamina propria and sub-mucosa and an irregular reduction of mucus cells (Prescott, Barker, Manninen and Miniats, 1981).

Interestingly, with the increasing use of rectal biopsy in human gastroenterology jejuni/coli GC have been reported as a cause of colitis and proctitis. The lesions are characterised by localized crypt abscesses of variable intensity, edema and sometimes a flattened epithelium depleted of goblet cells. Sequential observation of rectal mucosa showed that the lesions have resolved after a month (Price, Jewkes and Sanderson, 1979; Willoughby, Piris and Truelove, 1979; Duffy, Benson and Rubin, 1980).

#### Campylobacter infections in gnotobiotic ruminants

The main purpose of these studies was to investigate whether campylobacters are or are not pathogenic for neonatal calves. Since it is known that gnotobiotic animals have a relatively less developed defence systems or have been shown to be susceptible to agents not pathogenic under conventional circumstances it was thought that the gnotobiotic ruminants would be a suitable model to study the pathogenicity of Campylobacter spp. per se.

Previous work has suggested that conventional lambs develop a mild enteric disease after oral infection with Campylobacter spp., gnotobiotic lambs were therefore chosen as a possible more practical experimental animal to study not only the disease of lambs but as a model of calf infection.

Gnotobiotic ruminants have the advantage of being free from other enteropathogens which could interfere with the evaluation and interpretation of pathological change. They are also free from other bacteria or have a defined flora so it is technically easier to study

the distribution and quantify the colonization of campylobacters in different intestinal sites.

Three of the most common Campylobacter spp., C. jejuni biotype 1, C. coli and CHI type 1 from diarrhoeic calves, were selected from four A.D.R.A. outbreaks (Table 4.23). Strains of C. jejuni biotype 1 of different origin were included to compare their pathogenicity.

It has been suggested that after several passages in artificial media some campylobacter strains might lose cytotoxicity (Gubina, Zajc-Satler, Dragas, Zeleznik and Mehle, 1982) and inversely it was demonstrated that animal passage enhances virulence (Kazmi, Roberson and Stern, 1983). The strain used to inoculate the first calf had been subcultured many times in order to classify, clone and store it. In later experiments inoculation was performed with strains re-isolated from the first gnotobiotic calf; these strains were recovered as pure culture, were directly frozen from the primary isolation plates and when required used to inoculate BSA which was incubated and fed to the animals. All such strains used in the experiments had only one animal passage.

CHI and C. jejuni have both been associated with proliferative enteropathies in one or other animal species and a morphologically similar disease has been described in sheep; experiments with these Campylobacter spp. were carried out to assess whether campylobacter enteritis or proliferative enteropathies could be produced under these experimental conditions.

It has been suggested that the presence of flagella may be a requisite for adherence and colonization of some C. jejuni strains (McBride and Newell, 1983; Newell, McBride and Dolby, 1983). During

the experimental work it was discovered that one of the C. jejuni strains used in calves and lambs was composed of two variants, flagellate/motile (type "M") and aflagellate/non-motile (type "N") which could be differentiated by their colonial morphology in some culture media but remained undetected in the first experiments. Later, appropriate culture media was developed and adopted for the detection of both types of colonies which were separately enumerated in both faeces and intestinal sites.

Finally, differences in the pattern of infection have been described for different strains of jejuni/coli GC in mice. It is not clear whether all Campylobacter sp. or even all C. jejuni behave similarly in ruminants. These experiments were designed to allow microbiological assessment of possible differences in pathogenicity.

#### Materials and Methods

Animals and experimental design: Derivation of gnotobiotic calves and lambs, inoculation, necropsy and procurement of faecal samples were described in Chapter 2. Animals were maintained under gnotobiotic conditions in plastic isolators. Three gnotobiotic calves were infected with C. jejuni biotype 1 in 3 separate isolators. Eleven lambs were infected with either C. jejuni biotype 1, C. coli or CHI type 1 and were maintained in groups of 1, 2 or 3 animals in 6 isolators. Details of the identification of the animals and isolators, duration of the experiments, age of animals and campylobacter strains used are shown in Table 6.1.

Clinical monitoring: The animals were examined daily for their appearance, appetite and consistency and mucus contents of the faeces. The animals were fed reconstituted evaporated cows' milk and the volume

of feeding was increased gradually to a schedule based on age. Calves were fed three times a day as follows: 2 litres up to the 7th day of life, 2.5 litres up to the 10th, 3 litres up to the 14th and 3.5 litres up to the end of the experiment (Appendix No. 1). Lambs were fed following the standard milk intake schedule shown in Appendix No. 2. Milk intake of the calves and some lambs was recorded in the gnotobiotic unit and the available data were compared with the standard milk intake schedule of the A.D.R.A. Institute. The presence of mucus in faeces was recorded for each sample by observing its consistency and solubility in PBS.

Bacteriological procedures: Campylobacter methodology will be detailed in the appropriate experiment. Detection of other organisms was made by placing rectal swabs in NB, TM or Sabouraud liquid medium (SLM) and incubating them at  $37^{\circ}\text{C}-\text{O}_2$  for up to 10 days. Bottles were examined daily and if growth was observed subcultures were made as follows: NB was subcultured on a CBA plate which was incubated at  $37^{\circ}\text{C}-\text{O}_2$  for 1, 2 or 3 days as required; TM was subcultured on a CBA plate and incubated at  $37^{\circ}\text{C}-\text{ANA}-\text{O}_2$  for 3-5 days and SLM was subcultured on Sabouraud plates and incubated at  $37^{\circ}\text{C}-\text{O}_2$  for up to 7 days.

Miles and Misra's counts were performed on different selective media for campylobacters and on CBA or BABA plates incubated at  $37^{\circ}\text{C}-650\text{H}_2$ ;  $43^{\circ}\text{C}-550\text{H}_2$ ,  $37^{\circ}\text{C}-\text{ANA}-\text{O}_2$  or  $37^{\circ}\text{C}-\text{O}_2$  for 18 or 48 hours as required. Logarithmic values were calculated from the arithmetic mean of 2-6 replicates of Miles and Misra's colony counts. For statistical comparisons the logarithmic value of each replicate was separately calculated. Bacterial enumeration was considered to be negative when microorganisms were not isolated from the same sample by direct culture.

If a null Miles and Misra's count was obtained but the bacterium was isolated from non-quantitative culture, the logarithmic number of microorganisms was estimated and varied according to the number of negative Miles and Misra's plates as follows:  $<2.70$  (1 plate),  $<2.40$  (2 plates),  $<2.22$  (3 plates),  $<2.10$  (4 plates),  $<2.00$  (5 plates) and  $<1.92$  (6 plates).

Liver, spleen, lung and MLN were aseptically homogenized in an MSE homogeniser, then inoculated onto campylobacter selective media which were incubated at  $37^{\circ}\text{C}-650\text{H}_2$  or  $43^{\circ}\text{C}-550\text{H}_2$  as required and onto 3 CBA plates which were incubated at  $37^{\circ}\text{C}-\text{O}_2$ ,  $37^{\circ}\text{C}-\text{ANA}-\text{O}_2$  and  $37^{\circ}\text{C}-650\text{H}_2$  or  $43^{\circ}\text{C}-550\text{H}_2$ .

Approximately 0.1 ml of blood was cultivated onto 3 CBA plates which were incubated at  $37^{\circ}\text{C}-\text{O}_2$ ,  $37^{\circ}\text{C}-\text{ANA}-\text{O}_2$  and microaerophilically as required for the relevant Campylobacter spp. Two and 4 ml of blood were also inoculated into bottles containing 50 ml of BSA medium and incubated for up to 3 days at  $43^{\circ}\text{C}-550\text{H}_2$  or 5 days at  $37^{\circ}\text{C}-650\text{H}_2$ ; if growth was observed subcultures onto CBA and campylobacter selective media and CBA were made and plates were microaerophilically incubated as required.

Identification of other microorganisms than campylobacters was carried out by P. Wooding, University of Edinburgh.

Histology: These studies were restricted to the gastrointestinal tract, haemotoxylin-eosin sections of Ab, sites 1-5, Co, Ca and Re were prepared and examined for all gnotobiotic animals unless otherwise stated. PAS and silver-stained sections were examined in some gastrointestinal sites which will be specified in each experiment.

Scanning and transmission electron microscopy: TEM and SEM studies



were made on some intestinal sites at which lesions or high numbers of campylobacters were found.

Serology: Blood samples were taken before necropsy, sera separated and plate agglutination tests carried out as described in Chapter 2.

Statistics: Campylobacter logarithmic counts of gastrointestinal segments which were normally distributed were compared by one-way Analysis of Variance (Snedecor, 1950). If the differences were significant at  $p < 0.01$  the data were further analysed by Duncan's Multiple Range test (Harter, 1960) to determine the significant subsets. When the data were restricted to comparison of two gastrointestinal segments Student's t test was used (Downie and Heath, 1974). Non-parametric values were usually compared by the Kruskal-Wallis test (Sokal and Rohlf, 1981) but on occasion estimated values were considered as real counts. In all cases colonization of gastrointestinal segments was considered to be significantly different at  $p < 0.01$ .

## CHAPTER 6: SECTION A

Campylobacter jejuni infections in gnotobiotic calves

These experiments were carried out in order to determine whether a C. jejuni biotype 1 strain is enteropathogenic per se for gnotobiotic calves. Additional information was obtained on campylobacter colonization of different gastrointestinal segments at three PI periods, campylobacter excretion rates and the characteristics of the re-isolated strains.

Materials and Methods

Animals: Three gnotobiotic calves identified as No. 1 (Friesian breed), No. 2 (Aberdeen Angus breed) and No. 3 (Ayrshire breed) were used in three separate experiments (Table 6.1). A non-inoculated conventional calf of 8 days of life was used as control for histopathological studies.

Campylobacter oral inoculations: The inoculum was prepared, enumerated and administered to calves as described in Chapter 2. All calves were inoculated within the first 20-24 hours of life.

- Calf No. 1: was inoculated on the 3rd of September 1982 with  $2.025 \times 10^9$  ( $\log_{10} = 9.31$ ) bacteria of C. jejuni biotype 1 strain No. 476/82-D532-2, isolated from a diarrhoeic calf from A.D.R.A. outbreak D523 subgroup C1 (Tables 4.23). No identification of colonial types was performed.
- Calf No. 2: was inoculated on the 27th of October 1982 with  $2.25 \times 10^9$  ( $\log_{10} = 9.35$ ) and  $9.25 \times 10^9$  ( $\log_{10} = 9.96$ ) bacteria of types "M" and "N" respectively of C. jejuni biotype 1 strain No. 1279/82-D523-2, which had been re-isolated from calf No. 1 at the 5th day PI.
- Calf No. 3: was inoculated on the 15th of June 1983 with  $3.5 \times 10^9$  ( $\log_{10} = 9.54$ ) and  $7.5 \times 10^8$  ( $\log_{10} = 8.87$ ) bacteria of types "M" and "N" respectively of C. jejuni biotype 1 strain 1267/82-D523-2, which

was re-isolated from calf No. 1 at the 2nd day PI.

Necropsy: Examination and sampling was carried out as described in Chapter 2, except that rectum was not sampled in calf No. 1. Calves No. 1, 2 and 3 were killed at the 7th, 14th and 21st day PI respectively (Table 6.1).

Bacteriological techniques: All cultures for isolation and enumeration of campylobacters were incubated at  $43^{\circ}\text{C}-550\text{H}_2$  for 20 hours. Blood cultures were incubated at  $43^{\circ}\text{C}-550\text{H}_2$  for up to 3 days. Blood and homogenates of spleen, liver, lung and MLN were inoculated onto PN plates.

In calf No. 1 faeces were daily cultivated onto PN and CBA plates without performing bacterial enumeration. Miles and Misra's counts of inoculum and gastrointestinal sites were performed in duplicate on BABA and PN plates.

In calves No. 2 and 3 Miles and Misra's counts of the inoculum, faeces and gastrointestinal sites were performed on "Old PN", BABA, PN-CBA and/or CBA plus 0.2 per cent (w/v) of Oxoid Bacteriological agar L11 (CBAA). "Old PN" medium consists of PN plates stored for 20-30 days at  $+4^{\circ}\text{C}$  before use. "Old PN", PN-CBA and CBAA were used together or alternatively to detect type "M" or "N" of C. jejuni colonies. In calf No. 2 daily faecal counts and in calf No. 3 daily or every other day faecal counts were performed. Logarithmic values of types "M" or "N" of C. jejuni biotype 1 and contaminant bacteria were calculated from the arithmetic mean of Miles and Misra's colony counts on the available plates suitable for detection of the two colony types, which varied between 2 and 4 replicates. Total C. jejuni biotype 1 counts were obtained calculating the logarithmic value of each replicate which

varied between 4 and 6.

Histological examinations: These were carried out as described in Chapter 2, except that Re was not sampled in calf No. 1. Kerr's and Young's sections were examined in all gastrointestinal sites of calves No. 1 and 2. In calf No. 3 only Young's sections of site 1, Co, Ca and Re were examined. Periodic acid Schiff (PAS) stain was used for detailed examinations of the Co of calf No. 2.

Electron microscopy examinations: TEM studies were carried out at sites 3, 5 and Ca of calf No. 1. SEM studies were carried out at site 5, Co and Ca of calf No. 3.

- Processing of C. jejuni types "M" and "N" cultures for SEM: cloned cultures of both types were cultivated onto CBA plates and incubated at  $43^{\circ}\text{C}$ -550H<sub>2</sub> for 18 hours. Immediately after opening the jars campylobacters were fixed by gently flooding the plates with 2.5 per cent of glutaraldehyde in 0.1M PBS (pH 7.2) for 1 hour at room temperature. Growth on pieces of agar 10 mm in diameter was rinsed in 0.1M PBS containing 2 per cent dextrose and then post-fixed in 1 per cent osmium tetroxide in 0.1M PBS for 1 hour at room temperature. The agar blocks were dehydrated in graded acetones (30, 50, 80 and 3 times in 100 per cent), critical point dried and coated with gold palladium in a Polaron sputter coater. Specimens were examined in a Jeol T300 scanning electron microscope at 25 KV. Cultures were prepared for SEM at the A.D.R.A., Moredun Institute by Mr. E.W. Gray and his staff.

Faecal smears: Carbol-fuchsin faecal smears were made for the detection of campylobacters in the 3 calves. In calves No. 1 and 2 Giemsa smears were made following the technique described in Chapter 2. This latter technique was used to detect cells in faeces which were

enumerated and identified as polymorphs, lymphocytes or epithelial cells.

Serology: C. jejuni and C. coli plate agglutination tests were carried out as described in Chapter 2. PHA (Penner and Hennessy, 1980) of C. jejuni biotype 1 strains 1644/82-D523-2-Co types "M" and "N" were carried out by Dr. L. Roberts in The North of Scotland College of Agriculture, Aberdeen.

## Results

### 1.- Clinical observations

The three calves did not show sign of depression or illness throughout the experiments and diarrhoea was not detected. Feed consumption was normal (Table 6.2). In calf No. 1 the standard milk intake schedule was recorded. Calf No. 2 was fed with 1.620 litres less volume of milk than the standard amount but this difference was not significant when compared by Student's test with the expected feeding volume (Table 6.2,  $t_{(30)} = 0.603$ ). Calf No. 3 was fed with 1.130 litres more than the standard schedule.

1.1 Calf No. 1: the faeces were soft and had a mucous consistency from the 1st day PI until the end of the experiment.

1.2 Calf No. 2: the faeces were soft and contained mucus from the 1st until the 7th day PI (Figure 6.1). At the 8th and 9th day PI the faeces became notably softer, contained some mucus clots and stained the peri-anal zone. At the 10th and 11th day PI the faeces did not contain mucus, had a firm consistency and the faeces of the peri-anal zone were dry. At 13th and 14th day PI the faeces became soft again and contained mucus.

1.3 Calf No. 3: at the 2nd day PI the faeces contained red blood and

strings of fresh blood were seen at the 3rd and 4th day. At the 6th and 8th day PI the faeces became softer, acquired a notable mucous consistency and contained white "pseudomembranes" of mucus and strings of red blood on the surface. The mucoid consistency remained until the sacrifice of the animal but blood was not further noted.

## 2.- Bacteriological examinations

### 2.1 Detection of *C. jejuni* biotype 1 types "M" and "N" variants

When performing Miles and Misra's colony counts of the inoculum for calf No. 2 on "Old PN" plates the presence of two types of colonies was noted: one was a spreading large, flat colony of mucoid appearance (type "M") and the other was a small, non-spreading colony of entire edges (type "N"). These two colonial types could be distinguished on "Old PN" (Figure 6.2), PN-CBA or CBAA media but remained undetected on BABA or PN plates used in the experimental work with calf No. 1. It was possible to identify and enumerate these two types of colonies on appropriate culture media by the Miles and Misra's technique (Figure 6.3). SEM photographs showed that type "M" campylobacters were flagellated (Figure 6.4) whilst type "N" were mainly aflagellated (Figure 6.5). It was also observed that type "N" cultures formed more coccal forms than type "M" cultures at 18 hours incubation (Figure 6.6.). These coccal forms were capsulated (Chapter 5, Section B).

Six colonies of type "N" and six colonies of type "M" were cloned from the Ab, Co and Re of calves No. 2 and 3 and were studied in more detail. These strains were all classified as *C. jejuni* by the screening scheme (Chapter 5, Section A). It was noted that some cultures seemed to be stable "M" or "N" although most "M" type cloned cultures produced on subsequent subcultures a few "N" type colonies. Two stable "M" and

"N" variants of strain 1644/82-D523-2-Co, isolated from the colon of calf No. 2, were serotyped by the Penner and Hennessy PHA test and were both identified as serotype 4, 13, 16, 34, 43, 50, 26, indistinguishable from the original strain used to inoculate calf No. 1 (Table 4.23).

## 2.2 Calf No. 1

This animal remained uncontaminated during the experiment and the inoculated campylobacter was the only bacterium present. From the 2nd day PI onwards campylobacters were daily re-isolated and detected by carbol-fuchsin smears in the faeces. C. jejuni biotype 1 enumeration ( $\log_{10}/g$ ) in gastrointestinal segments on duplicate BABA and PN plates at 7th day PI, are shown in Table 6.3 and the C. jejuni colony counts obtained from two PN plates are summarized in Figure 6.7. All gastrointestinal segments were populated by C. jejuni biotype 1 with the numbers in the contents and paramucosal tissue of the large intestine  $10^2$  to  $10^4$  times greater than in the small intestine and Ab. Blood, lung, spleen, liver and MLN cultures were negative.

## 2.3 Calf No. 2

The animal was maintained free from contaminants until the 3rd day PI. On the 4th day PI and thereafter Bacillus spp. was isolated from faeces at approximately  $10^8$  to  $10^9/g$ . At post-mortem Bacillus spp. was isolated from all gastrointestinal segments (Table 6.5B) but spreading colonies prevented exact enumeration. From the 12th day PI Staphylococcus epidermidis was recovered from faeces but this bacterium was not detected by Miles and Misra's colony counts of either faeces or gastrointestinal segments and was probably present in low numbers.

C. jejuni was excreted at a constant level throughout ( $\bar{x} = 9.10 \pm 0.70$ ) from the 1st day PI onwards however, type "N" colonies were more



numerous from day 1 to 5 PI and on day 13 PI, whereas in the other days of the experiment type "M" colonies were predominant (Table 6.4 and Figure 6.11). From the 2nd day PI onwards campylobacters were microscopically detected in 9 out of 14 daily smears.

C. jejuni enumeration ( $\log_{10}/g$ ) in gastrointestinal segments on PN, PN-CBA and BABA at 14th day PI are shown in Table 6.5A, relative numbers of types "M" and "N" colonies are shown in Table 6.5B and Figure 6.8. The number of campylobacters in the large intestine was  $10^3$  to  $10^4$  and  $10^2$  times greater than in the small intestine and Ab respectively. Type "N" colonies predominated over type "M" colonies in all sites except in Re.

Campylobacter spp. and Bacillus spp. were isolated from lung, spleen, liver and MLN. Types "M" and "N" colonies were detected in all organs but in all sites type "N" was predominant over type "M" colonies. Few Staphylococcus epidermidis colonies were isolated from the MLN. Blood cultures were negative.

#### 2.4 Calf No. 3

Staphylococcus epidermidis was isolated from faeces from the 1st day of life before inoculation up to the 3rd day PI. This bacterium colonized poorly and was only detected by colony counts at day 0 and 1 PI (Table 6.6). At 13th day PI the animal was contaminated with Bacillus spp. and Clostridium welchii\* which were isolated from faeces up to the end of the experiment (Table 6.6). These contaminants more successfully colonized the large ( $10^7$  to  $10^8/g$ ) than the small intestine ( $10^2$  to  $10^6/g$ ) (Table 6.7B and Figure 6.10).

The mean logarithmic number of C. jejuni excreted from the 1st day PI was 8.95/g ( $\pm 1.00$ ) (Table 6.6). Types "M" and "N" of C. jejuni

\* Although frequently used in the literature Cl. welchii is now incorrect terminology and should be replaced by Cl. perfringens.

colonies were enumerated in faeces (Table 6.6 and Figure 6.12). The excretion of "N" type colonies predominated over "M" type, except at 6th and 10th day PI. Campylobacters were detected in all carbol-fuchsin faecal smears.

C. jejuni enumeration ( $\log_{10}/g$ ) in gastrointestinal segments on six selective plates are shown in Table 6.7A. The number of campylobacters in the large intestine was  $10^7$  to  $10^8$  whereas the numbers in the small intestine was low ( $10^2$  to  $10^4$ ). C. jejuni type "N" colonies predominated over type "M" colonies in Ab, sites 1, 2 and 3 and Co. In Ca and Re similar numbers of both colony types were enumerated (Table 6.7B and Figure 6.9).

C. jejuni, Bacillus spp. and Cl. welchii were isolated from the MLN. Cultures of blood, liver, spleen and lung were bacteriologically negative. Similar numbers of colonies of both "M" and "N" types of C. jejuni were obtained from the MLN.

## 2.5 Comparison of C. jejuni biotype 1 colonization of gastrointestinal segments of all calves

In small intestinal sites, Co and Ca, the number of campylobacters decreased with time (Tables 6.3, 6.5A and 6.7A; Figures 6.7, 6.8 and 6.9). These differences were statistically significant in all sites at  $p < 0.01$  except in site 2 of calves No. 1 and 2 and in Co of calves No. 2 and 3 (Table 6.8). The regression of the numbers of the small intestine on time was negative, linear and significant ( $F_{(1,13)} = 26.98$ ,  $p < 0.01$ ).

The Ab was colonized by  $10^5$  to  $10^6$  campylobacters per gram at statistically significant different levels in the 3 calves ( $p < 0.01$ , Table 6.8). The highest number of C. jejuni was detected in calf No. 2

(Table 6.5A,  $\bar{x} = 6.26$ ) and the lowest in calf No. 3 (Table 6.7A,  $\bar{x} = 5.21$ ).

Significantly higher number of C.jejuni ( $t_{(8)} = 7.962$ ,  $p < 0.001$ , Table 6.8) was detected in the Re of calf No. 2 (Table 6.5A,  $\bar{x} = 8.62$ ) than in calf No. 3 (Table 6.7A,  $\bar{x} = 7.94$ ).

### 3.- Pathological studies

#### 3.1 Gross findings

There were no gross abnormalities in the alimentary tract of any of the calves. The stomachs contained clotted milk and the small intestine normal fluid digesta. In all three calves the contents of Co, Re and Ca were mucoid and in the Co of calf No. 2 the contents had a notable gelatinous consistency (Figure 6.13). The mucosal surfaces of both the small and large intestine did not show any alteration although there was possibly some oedema of the wall of the large bowel. The MLN, liver, spleen and lung were normal.

#### 3.2 Histopathology

##### 3.2.1 Calf No. 1

Minor pathological changes were apparent in ileum and caecum only; all other sites were normal. In site 4 most villi contained at their tips bloated pigmented macrophages as well as eosinophils; they were otherwise rather acellular and the villi and crypts were normal. The only abnormality in site 5 was migration of some neutrophils throughout the epithelium of the "M" cell zones above Peyer's patches and in local capillaries.

In some areas of the Ca a diffuse infiltrate of mononuclear cells and neutrophils was present in the surface epithelium and sometimes in groups in the lumen (Figure 6.14). Incipient or early crypt abscesses

were present in the lumen; here the epithelial cell lining showed no alteration (Figure 6.15). In advanced cases the crypt epithelium was damaged and the surviving cells flattened. These crypt abscesses contained abundant polymorphs in the lumen with inflammatory exudate in the surrounding lamina propria (Figure 6.16) which sometimes extended through the muscularis mucosae (Figure 6.17).

Silver stained campylobacters were occasionally found in small groups or isolated, generally close to the mucosal surface but not attached, in the lumen and crypts of the Ca and Co. They were rarely found in the lumen of the small intestine. No bacteria appeared to be intracellular.

### 3.2.2 Calf No. 2

The most notable pathological changes were detected in Co and Re; all other gastrointestinal segments were normal and only minor alterations were noted. In Ab one small area had infiltrates of mononuclear cells and a few neutrophils between the glands. The small intestine had essentially normal morphology throughout although most sites contained a few tiny aggregates of neutrophils in the lamina propria and in some local capillaries. The small intestinal sites contained a few eosinophils which increased in numbers distally.

The Ca was normal throughout except for scattered focal collections of neutrophils in the lamina propria. The Co showed some oedema and contained few foci of mononuclear cells and neutrophils localized in an epithelium flattened and devoid of goblet cells. In affected areas adherent mucus overlay the mucosal surface (Figures 6.18, 6.19 and 6.20B). Such changes were absent in control conventional calf of similar age (Figure 6.20A). In Re a definite proctitis was found with light

aggregates of neutrophils in the lamina propria often migrating through the surface epithelial cells (Figures 6.21 and 6.22).

Silver stained campylobacters were rarely found in the lumen of the small intestine. In Ca, Co and especially Re high numbers of campylobacters were seen in the lumen colonizing the para-mucosal area, often included in a mucus-like material and some were seen close to goblet cells. No campylobacters were detected intracellularly.

### 3.2.3 Calf No. 3

No alterations were noted in the small intestine. In site 1 macrophages, lymphocytes and goblet cells were quite numerous, both in villi and in lamina propria of the lower mucosa. The other small intestinal sites were normal.

In Ca and Co aggregates of macrophages and eosinophils were detected in the lamina propria and there was some separation of the glands. Occasionally, the epithelium overlying these aggregates was cuboidal. Neutrophils were virtually absent and the epithelium of the glands appeared normal. The Re was similar, except that mononuclear cell aggregates were discrete and almost invariably covered by cuboidal epithelium. There was no evidence of hypertrophy of the large bowel mucosa.

Silver stained bacteria including a few campylobacter-like-organisms were occasionally seen in the luminal paramucosal area of site 1, Co, Ca and Re. No campylobacters appeared to be intracellular.

### 3.3 Ultrastructural studies

TEM ultrastructure of site 5 of 8-day-old calf No. 1 did not differ markedly from that described for the normal 48-hour-old gnotobiotic calf (Mebus, Newman and Stair, 1975a). The only abnormalities were

the occasional presence of neutrophils migrating through the epithelium and more frequently lymphocytes and neutrophils in the lumen (Figure 6.23). Occasionally remnants of epithelial cells and few erythrocytes were also seen in the lumen. Microvilli were well developed in all areas and mitochondria showed a normal morphology. The large cytoplasmic vacuoles of the very young germ-free animal in the middle and apical epithelial cells of the villi were absent. Campylobacters were found neither intracellularly nor near the luminal surface of the villi. Occasionally, isolated groups of campylobacters were found in the lumen of deep crypts. They were invariably encircled by an amorphous mucus-like material but showed no close association or attachment with the microvilli of host cells.

TEM examinations of the Ca of calf No. 1 corroborated the histopathological findings. Occasionally neutrophils were found migrating through the epithelium or within the lumen of some crypts (Figure 6.24). Most crypts did not have these cells but frequently isolated (Figures 6.25, 6.26 and 6.27) or rarely groups (Figures 6.28 and 6.29) of campylobacters were found in close proximity with the villus surface and always included in an amorphous mucus-like material. On one occasion only the apparent remnants of damaged bacteria attached and partially encircled by the cell membranes of an epithelial crypt cell was observed (Figures 6.26 and 6.27). Intracellular campylobacters were not found.

SEM examinations of site 5, Co and Ca of calf No. 3 failed to detect any campylobacters on the surface (Figure 6.32) or included in mucus material (Figure 6.33). The morphological characteristics of the epithelial associated lymphoid follicles (Peyer's patches) of site

5 were considered to be similar to those of the normal 2-day-old gnotobiotic calf (Torres-Medina, 1981) except for the presence of more M-cell domes which were clearly visible on the surface of the specimen (Figures 6.30 and 6.32). The colonic mucosa had less marked ridges and furrows than that described in a normal 2-day-old gnotobiotic calf (Mebus, Newman and Stair, 1975a) but otherwise no other alterations were noted. In Ca and Co numerous mucus aggregates were examined and other bacteria different from campylobacters were found included in the mucus, probably Bacillus spp. or Cl. welchii (Figure 6.33).

#### 4.- Detection of cells in faeces by Giemsa faecal smears

Tables 6.9A and B show the approximate number of cells in faeces and the relative proportion of polymorphonuclears, lymphocytes and epithelial cells. In calf No. 1 cells were numerous from the 2nd day PI onwards and in calf No. 2 cells were found in considerable numbers on the 3rd, 4th, 5th, 8th and 9th day PI. The relative proportion of cells was obtained where it was possible to enumerate 10 or more cells. Polymorphonuclears varied between 48.4 and 80.6 per cent, lymphocytes between 5.6 and 14.9 per cent and epithelial cells between 11.6 and 45.2 per cent. Interestingly, an increase of the number of cells was accompanied by a greater relative proportion of polymorphonuclears.

#### 5.- Serological examinations

Attempts to perform plate agglutination tests with C. jejuni biotype 1 strains 1644/82-D523-2-Co, types "N" and "M", re-isolated from the Co of calf No. 2, were unsuccessful due to autoagglutination. The roughness could not be eliminated by dilution of the antigens with 0.5 per cent formol PBS.



As a second choice calf sera were titred against strain C. jejuni biotype 1 1744/81-B2464-2, isolated from an A.D.R.A. calf neonatal diarrhoea outbreak (Table 4.23) and used to infect gnotobiotic lambs (this Chapter, Section B). This strain shares six Penner's antigens with the inocular strain (Table 4.23). Agglutination antibody was detected in calf No. 3 at low titre (1/40) but the sera of calves No. 1 and 2 did not agglutinate (less than 1/5).

Alternatively agglutination tests were also carried out with C. coli antigen of strain 569/82-D531-32 isolated from another A.D.R.A. calf neonatal diarrhoea outbreak (Table 4.23) and used to infect gnotobiotic lambs (this Chapter, Section B). The sera of the three gnotobiotic calves did not agglutinate (less than 1/5).

### Discussion

Other investigators have demonstrated that gnotobiotic calves are susceptible to enteric disease and diarrhoea can be produced within 40 hours with rotavirus (Mebus and Newman, 1977) and coronavirus (Mebus, Newman and Stair, 1975b). Under the conditions of these experiments highly susceptible animals have been inoculated with a strain of C. jejuni biotype 1 originally isolated from an outbreak of neonatal diarrhoea. All were successfully colonized but did not show any sign of general illness, anorexia or diarrhoea. Instead, a mild disease was produced characterised by minor clinical and pathological changes. The high cost of these experimental animals prevented the use of control non-infected gnotobiotic calves, so the pathological changes had to be compared with the available descriptions which are restricted

to 2-day-old normal gnotobiotic calves (Mebus, Newman and Stair, 1975a; Torres-Medina, 1981). Another limitation of this work was the use of only one strain of C. jejuni as most studies indicate different virulence of strains (vide supra).

A constant feature in the three calves was the mucoid nature of the faeces. The normal faecal texture of artificially milk-fed calves is soft but in calves No. 2 and 3 the consistency was frankly softer on the 8-9th and 6-8th day PI respectively. These changes were not noted in calf No. 1. Visible red strings of blood were noted only in calf No. 3 at the 2nd-4th day PI. Mucoid consistency and occult or visible blood in faeces have been reported in previous experiments in conventional calves infected with jejuni/coli GC (Jones and Little, 1931b; Al-Mashat and Taylor, 1980b and Firehammer and Myers, 1981) and the presence of mucus in calf No. 1, mono-infected with C. jejuni corroborates that this bacterium is responsible for its release. The presence of mucus was notable in the lumen of the colon of calf No. 2 which showed areas of epithelium practically devoid of goblet cells. Similar observations of discharging goblet cells have been cited in campylobacter colitis of gnotobiotic dogs (Prescott, Barker, Manninen and Miniats, 1981) and humans (Willoughby, Piris and Truelove, 1979) although the lesions of the colon of calf No. 2 differ in degree from the marked mucosal thickening and oedema of gnotobiotic dogs and humans (Price, Jewkes and Sanderson, 1979). Sequential rectal biopsy in humans shows that there is a recovery which is partial 5 days after the onset of disease and complete after a month; the histology of the Co of calf No. 2 at the 14th day PI is similar to the appearance of human rectum in an early recovery stage (Willoughby, Piris and Truelove,

1979) and the colon of calf No. 3 at the 21st day PI showed a normal number of goblet cells.

Cholera toxin has been shown to specifically cause mucus release from goblet cells (Elliott, Carpenter, Sack and Yardley, 1970) so the recent discovery of a cholera-like toxin in jejuni/coli GC (Ruiz-Palacios, Torres, Torres, Escamilla, Ruiz-Palacios and Tamayo, 1983) may suggest that this type of enterotoxin is responsible for the presence of mucus in the faeces of these calves. Non-specific antigen-antibody immune complexes induce mucus release in duodenal segments of rats but their action is less marked than cholera toxin (Walker, Wu and Bloch, 1977). Considerable evidence suggests that mucus protects the mucosal surface against penetration by microorganisms and toxins and reduces adherence of bacteria (Lake, Bloch, Neutra and Walker, 1979) and campylobacters (McCardell, Madden, Bier, Lee and Dallas, 1983). In the case of these microorganisms, which are mucosa associated (vide supra), the presence of crypts filled with mucus may represent an ecological advantage for the bacterium. Interestingly, in this work very frequently when a campylobacter organism or group were seen in the lumen of the intestine by silver stains or TEM they were included into an amorphous mucus-like material and sometimes found near to goblet cells. Similarly, Al-Mashat (1981) found campylobacter-like-organisms very close to goblet cells in the caecal crypts of jejuni/coli GC inoculated conventional calves. Furthermore, Prescott, Barker, Manninen and Miniats (1981) found in gnotobiotic dogs inoculated with C. jejuni occasional goblet cells which were extruding mucus and seemed to have microorganisms included within the mucus droplet. This work corroborates these observations and shows that C. jejuni also behaves

in calves as a mucosa-associated microorganism although SEM failed to detect campylobacters included in the mucus layer as has been described in rodents (Field, Underwood, Pope and Berry, 1981). In the present study SEM fixation of tissues was carried out according to the method used for rodents (Pope, Cole, Guentzel and Berry, 1979) and only small fragments of mucus were obtained instead of layers. Detailed SEM observations of these mucus fragments failed to detect campylobacters although other bacteria were found in the mucin. The absence of a mucus layer in calves may indicate a different texture of the mucin in these animals.

Faecal samples were collected from the walls of the rectum except on three occasions from calf No. 2, on the 4th, 10th and 14th day PI. In these cases samples were obtained from faeces that had just been voided. Interestingly, in the 3 samples a decrease in the number of campylobacters was detected which may indicate that colonization in the paramucosal area is more efficient than in luminal contents.

Higher numbers of campylobacters were found in the large than in the small intestine showing that this microorganism prefers to colonize the lower portion of the alimentary tract of gnotobiotic calves. This finding is similar to the other results from gnotobiotic calves (Morgan, Hall, Reynolds and Parsons, 1983), piglets (Andress, Barnum and Thomson, 1968; Kashiwazaki, Namioka and Yabiki, 1971), dogs (Prescott, Barker, Manninen and Miniats, 1981) and neonatal rodents (Field, Underwood, Pope and Berry, 1981). In conventional mice it has been demonstrated that the number of C. jejuni is higher in the small than in large intestine but no quantitative information is available for conventional calves. Al-Mashat and Taylor (1980b) reported the

re-isolation of jejuni/coli GC from the large and small intestine but they did not performed bacterial enumeration. They found that jejuni/coli GC was isolated in large numbers from the ileum, , Ca and Co and lesser number of colonies were obtained from the Ab and jejunum of some animals.

When this work was undertaken the published information on experimental infections of conventional calves with jejuni/coli GC strain indicated that gross changes were confined to the lumen content of small and large intestine and the associated lymph nodes (Al-Mashat and Taylor, 1980b). In the first experiments (calf No. 1) of this study the content of the large intestine only was mucoid and gross lesions were not obvious. Histologically, it was found that lesions were mainly localized in the large intestine, and for that reason Re was included in subsequent studies. Only minor histopathological changes were observed in the small intestine of the calves with no important structural changes in the morphology. SEM observations of site 5 of 22-day-old calf (No. 3) demonstrated higher domes than described in a normal 2-day-old gnotobiotic calf (Torres-Medina, 1981) but this difference is most probably related to the age of the animals. The absence of vacuoles in the cytoplasm of the epithelial cells, normally found in 2-day-old gnotobiotic calves (Mebus, Newman and Stair, 1975a; Torres-Medina, 1981) is believed to be due to the age of the calves examined rather than being an abnormality, although this speed of maturation of the villus epithelium to the adult pattern may have been accelerated by the treatment received.

The most prominent lesions were found in the Ca, Co and Re in association with the highest numbers of campylobacter. The presence

of scattered crypt abscesses of different stages in the Ca of calf No. 1 is similar to the pathology found in the Co and Re in humans suffering from jejuni/coli GC enteritis (Price, Jewkes and Sanderson, 1979; Duffy, Benson and Rubin, 1980) and to that described in the ileum and to a lesser extent duodenum of experimentally infected conventional calves (Al-Mashat, 1981). Campylobacters were found isolated or in groups in the lumen of crypts but not in the cytoplasm of epithelial cells. In affected crypts epithelial damage was evident showing that campylobacters, the only bacteria present in this calf, were apparently capable of inducing lesions without actually invading the mucosa, probably by means of locally released cytotoxic factors. The presence of mucus in faeces and blood strings in one of the calves was consistent with the pathology. After 21 days of infection aggregates of macrophages and eosinophils replaced neutrophils in the large intestinal sites with a cuboidal epithelium covering the infiltrates; these lesions were mild indicating recovery from the disease. SEM observations of the Co mucosa of calf No. 3 revealed less marked ridges and furrows than that described in a normal 2-day-old gnotobiotic calf (Mebus, Newman and Stair, 1975a) and in calf No. 2 histopathological studies showed a flattened surface, possibly formed by a degree of underlying mucosal oedema, and with some goblet cell depletion on the surface. Unfortunately, the lack of gnotobiotic control calves of 14 and 21 days of life makes assessment difficult.

Jones and Little (1931b) and later Al-Mashat (1981) described mild gross and histological changes respectively in the Ab of calves infected with C. jejuni. In the present study the abomasal mucosa was normal in calves No. 1 and 3 and in calf No. 2 one small area of

mild cellular infiltration was found without damage of the epithelium despite the continued presence of C. jejuni.

Most of the pathological changes described in experimental infections of conventional calves by Al-Mashat (1981) and Al-Mashat and Taylor (1980b) were also found in these gnotobiotic calves although affecting different gastrointestinal segments. Aggregates of inflammatory cells, crypt abscesses, goblet cell discharge, degeneration of epithelial cells or presence of cuboidal epithelium are alterations caused by C. jejuni and were common findings in all these investigations. Other described lesions such as stunted villi or villous atrophy and loss of luminal epithelium were not observed although exfoliated cells were seen. Morgan, Hall, Reynolds and Parsons (1983) in experiments with gnotobiotic calves inoculated with mixtures of C. jejuni and C. coli found petechial haemorrhages and exfoliation of enterocytes in the colonic mucosa of animals examined after 2-4 weeks PI. The latter lesions were not found in the present studies.

In small intestinal sites, Ca and Co the number of campylobacters significantly decreases ( $p < 0.01$ ) with time PI. This decrease is more notable in the small than in the large intestine in which campylobacters remain at high levels at the 21st day PI (Figure 6.9, approximately  $10^8/g$ ). This phenomenon indicates that the environment of the small intestine is appropriate for colonization only during the first phase of infection or rather that immune effects first exert their action at this site.

In these three calves although serology was done with a heterologous strain, the antibody response was very poor reaching only a titre of 1/40 after 21 days of exposure. This coincides with other works on



Campylobacter spp. infections in gnotobiotic piglets in which low or no levels of antibody were detected up to the 14th day PI (Kashiwazaki, Namioka and Yabiki, 1971), 21st day PI (Andress, Barnum and Thomson, 1968) and 47th day PI (McCartney, Lawson and Rowland, 1984). These findings are quite different from the results obtained in experimental infections with conventional calves (Al-Mashat and Taylor, 1980b) and pigs (Olubunmi and Taylor, 1982) infected with jejuni/coli GC in which titres up to 1/640 were detected after the 10th to 16th day PI. The reason for this differences could lie in immune tolerance (Bourne and Newby, 1981), site of colonization and the influence of normal flora. Unfortunately the auto-agglutination of the homologous antigens could not be prevented despite the use of a higher concentration of formalin as recommended by Butzler (1978). Auto-agglutination is a common phenomenon among jejuni/coli GC strains (Butzler, 1978; Butzler and Skirrow, 1979; Bryner, Ritchie and Foley, 1982).

Interestingly, the number of campylobacters were significantly higher ( $p < 0.001$ ) in the Re of calf No. 2, in which a notable proctitis was found, than in calf No. 3 in which only mild lesions were detected. In Ab the number of campylobacters varied between  $10^5$  and  $10^6$  without showing the correlative decrease on time found in other intestinal segments, probably because colonization in this organ is directly influenced by ingestion of bacteria, dilution of milk and digestive processes.

The presence of Bacillus spp. and Bacillus spp. and Clostridium welchii in calf 2 and 3 respectively (Figure 6.10) apparently did not interfere with C. jejuni colonization. Interestingly, Staphylococcus epidermidis infection in calf No. 2 and 3 only survived

poorly. Contrasting with this, in experiments with gnotobiotic piglets (Kashiwazaki, Namioka and Yabiki, 1971), dogs (Prescott, Berker, Manninen and Miniats, 1981) and lambs (this Chapter, Section B) contamination with S. epidermidis reached high numbers in the gastrointestinal tracts. It is not known if this lack of colonization is due to the S. epidermidis strains studied here or a characteristic of gnotobiotic calves.

Blood cultures were negative but campylobacters were isolated from the MLN and visceral tissues of calf No. 2 and from the MLN of calf No. 3, indicating that transient bacteremia had occurred. In experiments with conventional calves Al-Mashat (1981) and Taylor and Al-Mashat (1984) found similar results but the bacteria disappeared more rapidly from extra intestinal sites. The isolation of campylobacters from internal organs at the 14th and 21st day PI probably indicates that the gnotobiotic calf is more susceptible to infection and bacteria persist more readily; this is supported by the recovery of Bacillus spp., Clostridium welchii and S. epidermidis from these tissues. The absence of campylobacters in blood in these experiments and Al-Mashat's contrast with the results of Bryner and Warner (1983) who used C. jejuni and C. coli strains isolated from ovine and swine fetuses respectively. Both strains were re-isolated from the blood of calves, C. jejuni for at least 42 days in one calf, 28 days in two, 14 days in one and shorter periods in others. These results may indicate differences in strains isolated from abortions but it has to be pointed out that the bacteriological procedure used by Bryner and Warner (Second Workshop on Campylobacter Infections, Poster Communication, 1983) is much more sensitive than those used in these and Al-Mashat's

experiments.

King (1957) described the isolation of a non-motile jejuni/coli GC strain and concluded that this isolate has occurred naturally. Later, Smibert (1974) described two types of colonies commonly found on primary isolation; one is flat, finely granular, translucent, with an irregular edge and spreads along the direction of the streak tending to swarm and coalesce; the other is round, 1-2 mm of diameter, raised, convex, entire with a slight opaque center. These two types of colonies are remarkably similar to the "M" and "N" described in this work. Skirrow and Benjamin (1980b) cited that occasionally strains threw off discrete non-effuse colonies, so that two colony types can co-exist in one culture. The evidence suggests that the "M" and "N" types of colonies encountered in a strain randomly selected for this experimental infections are relatively common and can remain undetected, as happened in calf No. 1, when the culture media used failed to reveal the typical colonial morphology. Black, Levine, Blaser, Clements and Hughes (1983) also described that one of the C. jejuni strains (No. A3004) used to infect human volunteers contained an aflagellated variant; colonies recovered from the stools of inoculated volunteers were all of spreading type (Black, Personal Communication, 1983).

Newell, McBride and Dolby (1983) found that an aflagellate/non-motile C. jejuni variant poorly colonized the intestinal tract of infant mice in comparison with the flagellate/motile wild strain. These authors described that the flagellate wild type successfully colonized for up to 30 days whereas the aflagellate variant could not be recovered after the 7th day PI. In contrast, in these present

experiments type "N" colonies (aflagellate/non-motile type) were more numerous than type "M" colonies (flagellate/motile) in most gastrointestinal segments of calves at the 14th and 21st day PI. In a few intestinal segments and in faeces on occasions "M" colonies equalled or exceeded "N" colonies. It is difficult to assess the significance of the distribution of the types of colonies on the pathogenesis of the disease especially as experimental work in ruminants with separated clones of both types has not been performed.

Identification of campylobacter-like organisms by Gram stains of faecal smears correlates well with isolation in cattle (Taylor and Al-Mashat, 1984) and in humans (Ho, Ault, Ault and Murata, 1982). Smears stained with aqueous basic fuchsin have 94 per cent of the sensitivity of culture and are superior to the Gram's technique for detecting vibrioid cells in human stools (Park, Hixon, Polhemus, Ferguson, Hall, Risheim and Cook, 1983). In the 3 calves carbol-fuchsin faecal smears demonstrated a sensitivity of 85.3 per cent (29/34) in detecting vibrioid organisms in bacteriologically proven campylobacter faeces ( $10^8$ - $10^9$  bacteria/g).

The presence of red cells and neutrophils in faeces is a common finding in more than 75 per cent of human patients with campylobacter enteritis (Blaser and Reller, 1981). Firehammer and Myers (1981) found a transient rise in faecal leukocytes 48 hours after challenge of a calf with jejuni/coli GC. In the present experiments the proportion of neutrophils was increased during the days in which more cells were excreted. These cells are probably generated in the site 5 and large intestinal segments where crypt cells aggregates were detected.

Kazmi, Roberson and Stern (1983) found that after 3 passages

C. jejuni became lethal to mice when re-inoculated by intraperitoneal route. In the present experiments one animal passage or successive subcultures on artificial media did not seem to substantially modify the pathogenicity of the strain studied although it was noted that the faeces of calf No. 1 had less soft consistency than that of other calves. Al-Mashat (1981) found that a strain of jejuni/coli GC was virulent for calves after 9 passages on culture media and reconstitution from freeze-dried stock.

## CHAPTER 6: SECTION B

### Campylobacter infections in gnotobiotic lambs

Oral inoculation of gnotobiotic lambs was carried out with two C. jejuni biotype 1, one C. coli and one CHI type 1 strains isolated from A.D.R.A. calf diarrhoea outbreaks (Table 4.23). The main purpose of these studies is to assess whether these bovine campylobacter strains are pathogenic per se for lambs, as an experimental model of the disease of calves. Additional information was obtained about colonization of gastrointestinal segments at different PI periods, campylobacter excretion rates and characteristics of re-isolated strains.

#### I) Campylobacter jejuni infections

##### Materials and Methods

Animals: five gnotobiotic lambs identified as follows: No. 1 (Black-face), No. 2 (Halfbred), No. 3 (Greyface), No. 4 and No. 5 (Suffolk). The animals were maintained in isolators as follows: one animal in isolator D and two animals in each of two isolators (E and F), as shown in Table 6.1.

Campylobacter oral inoculations: the inoculum was prepared, enumerated and orally administered as described in Chapter 2. All lambs were inoculated within 24 hours of birth.

- Lambs No. 1, 2 and 3: were inoculated with C. jejuni biotype 1, strain 476/82-D523-2, isolated from a diarrhoeic calf from A.D.R.A. outbreak D523, subgroup C1 (Table 4.23). In lambs No. 2 and 3 this strain contained only detectable type "N" colonies and it can be estimated that type "M" colonies may be present in numbers of less than  $10^5$ . In lamb No. 1 identification of colony types was not done.

Lamb No. 1 was inoculated on the 3rd of July 1982 with  $2.5 \times 10^8$  ( $\log_{10} = 8.40$ ) bacteria and lambs No. 2 and 3 were inoculated on the 28th of May 1983 with  $6.3 \times 10^8$  ( $\log_{10} = 8.80$ ) bacteria.

- Lambs No. 4 and 5: were inoculated on the 25th of May 1982 with  $3.87 \times 10^8$  ( $\log_{10} = 8.59$ ) bacteria of C. jejuni biotype 1 strain 1744/81-B2464-2 isolated from a diarrhoeic calf from A.D.R.A. outbreak B2464, subgroup A1 (Table 4.23).

Necropsy: examination and sampling was carried out as described in Chapter 2 except that Re of lambs No. 1, 4 and 5 was not sampled. Lambs No. 4, 1, 5, 2 and 3 were killed on day 3, 4, 7, 19 and 30 PI respectively (Table 6.1).

Bacteriological techniques: campylobacter cultures were incubated as described for gnotobiotic calves (this Chapter, Section A). In lambs No. 1, 4 and 5 faeces were cultivated daily onto PN and CBA plates without performing bacterial enumeration. In lambs No. 2 and 3 Miles and Misra's counts of faeces were carried out onto PN, "Old PN", PN-CBA and CBA plates.

In lamb No. 1, 4 and 5 enumeration of gastrointestinal segments was performed on BABA and PN plates; in lambs No. 3 and 4 PN, PN-CBA and CBA plates were used.

Logarithmic values of types "M" and "N" of C. jejuni biotype 1, contaminant bacteria and total C. jejuni counts were obtained as described for gnotobiotic calves (this Chapter, Section A).

Histological examinations: were carried out as described in Chapter 2, except that Ab and Re were not sampled in lambs No. 1, 4 and 5. Young's sections were examined in sites 1, 2, 3, 4, 5, Ca and Co of lambs No. 1, 4 and 5; sites 2 and 5, Ca and Co of lamb No. 2; and



sites 3 and 5, Ca and Co of lamb No. 3.

Electron microscopy examinations: TEM was performed at site 5 and Ca of lamb No. 5. SEM was carried out at site 5, Ca and Co of lambs No. 2 and 3.

Serology: plate agglutination tests were performed as previously described (Chapter 2) with antigens C. jejuni biotype 1 1744/81-B2464-2 and C. coli 569/82-D531-32.

## Results

### 1) Clinical observations

None of the lambs showed any apparent sign of depression or illness throughout the experiments. Milk intake was recorded up to the 4th day of life in lambs No. 1 and 4 and to the 8th day of life in lamb No. 5 (Appendix No. 3), and when compared with the expected milk intake for the corresponding period it was found that milk consumption was optimal (Table 6.10).

In the five lambs faeces were soft and had a mucoid consistency from 2nd day PI. The faeces became notably softer in lamb No. 3 at the 26th day PI but two days later they acquired the normal soft consistency. Visible blood was not detected in any of the samples.

### 2) Bacteriological examinations

#### 2.1 Detection of C. jejuni biotype 1 types "M" and "N" variants

The two types of colonies were detected and enumerated in gastrointestinal segments and faeces in lambs No. 2 and 3 following the same procedure described for gnotobiotic calves. Two colonies of type "N" and two of type "M" were cloned from the Co of lambs No. 2 and 3 and were classified as C. jejuni biotype 1 by the Screening Scheme (Chapter 5, Section A). In lambs No. 1, 4 and 5 these two types were not

distinguished during the experimental work because suitable media had not been used to evidence their typical morphology. Nevertheless, subsequent work with strains re-isolated from lambs No. 1, 4 and 5 demonstrated the existence of both types of colonies in both C.

jejuni biotype 1 strains.

## 2.2 Lamb No. 1

No other bacteria than campylobacter were isolated up to the 3rd day PI inclusive. At post-mortem, on the 4th day PI, Streptococcus spp. and Bacillus spp. were isolated and their enumeration in gastrointestinal segments is shown in Table 6.11B. Streptococcus spp. was found at levels of  $10^3$  bacteria/g in Ab, sites 2 and 3, not detectable levels in duodenum ( $< \log_{10} 2.40$ ),  $10^7$ /g in site 4 and  $10^8$ /g in Co and Ca. As Bacillus spp. formed spreading colonies no exact enumeration could be performed; this bacterium colonized the large intestine at levels of approximately  $10^6$  bacteria/g but was not detected in the small intestine.

C. jejuni biotype 1 was recovered from faeces from the 1st day PI onwards either on PN, CBA or TM. C. jejuni colonized all gastrointestinal segments but more successfully the large intestine ( $10^{10}$  bacteria/g) than the small intestine ( $10^3 - 10^7$  bacteria/g). The ileum reached higher levels of campylobacters ( $10^7$  bacteria/g) than any other small intestinal segment (Table 6.11A and Figure 6.34).

Blood cultures were negative. C. jejuni was isolated from the MLN, liver, lung and spleen. Bacillus spp. and Streptococcus spp. were isolated from the MLN. Very few colonies of Streptococcus spp. were obtained from lung cultures.

## 2.3 Lambs No. 2 and 3

Contaminant bacteria, E. coli and S. epidermidis, were simultaneously detected in both lambs from the 2nd day PI onwards. NB, TM and CBA and anaerobic cultures were negative at the 0 and 1st day PI indicating that these microorganisms established between 24 - 48 hours (Tables 6.12 and 6.14). E. coli was excreted in faeces at levels of  $10^2 - 10^5$  bacteria/g and S. epidermidis at  $10^8 - 10^{10}$ /g. At post-mortem E. coli was found in all gastrointestinal segments just on or below the lower limit of bacterial enumeration and S. epidermidis colonized the small intestine at levels of  $10^2 - 10^6$  bacteria/g and the large intestine at  $10^7 - 10^9$  (Tables 6.13B and 6.15B). In the old lamb (No. 3) S. epidermidis levels were lower than the counts of lamb No. 2 in all gastrointestinal segments except in Ab and site 5 (Figure 6.37), although the difference of the mean logarithmic numbers of the small intestinal sites of both lambs was not significant ( $t_{(8)} = 0.347$ ).

Campylobacters were isolated from lamb No. 2 from the 1st day PI and from lamb No. 3 from the 2nd day PI onwards. C. jejuni biotype 1 was excreted in faeces at levels of  $10^9 - 10^{10}$  bacteria/g from the 2nd day PI in both animals (Tables 6.12 and 6.14). The relative excretion of types "M" and "N" was variable. In lamb No. 2 type "M" colonies were more numerous than type "N" in all days PI at which enumeration was performed except the 2nd and 4th day PI (Table 6.12). In lamb No. 3 type "M" was more numerous on the 2nd, 5th to 9th and 22nd to 28th day PI whereas in the rest of the days the inverse relationship was found (Table 6.14, Figure 6.38). Campylobacters colonized all gastrointestinal segments being more numerous in Co and Ca ( $10^9 - 10^{10}$  bacteria/g) than in small intestinal segments ( $10^2 - 10^6$ /g); in Re intermediate levels were found ( $10^7$ /g) (Tables 6.13A and 6.15A).

Type "N" colonies were more numerous in all gastrointestinal segments except in Re of lamb No. 2 in which  $\log_{10}$  1.68 more type "M" than "N" colonies were enumerated and in site 2 of lamb No. 3 in which the number was below the resolution limit of the enumeration method (Tables 6.13B and 6.15B; Figures 6.35 and 6.36).

Blood cultures were negative. In lamb No. 2 C. jejuni types "M" and "N", S. epidermidis and small number of E. coli colonies were isolated from the MLN; C. jejuni type "M" but not "N" colonies were isolated from the spleen and liver; and S. epidermidis was also isolated from the lung, spleen and liver. In lamb No. 3 only S. epidermidis was isolated from MLN and liver.

#### 2.4 Lambs No. 4 and 5

E. coli was first detected in lamb No. 5 on the day of inoculation and from the 1st day PI onwards this bacterium was daily isolated from the faeces of both lambs. Streptococcus spp. was excreted in lamb No. 5 from the 1st day PI and in lamb No. 4 from the 2nd day PI onwards. High levels of E. coli ( $10^3$  to  $10^{11}$  bacteria/g) were detected in all gastrointestinal segments of both lambs. Streptococcus spp. colonized all gastrointestinal segments in less numbers than E. coli except in site 1 of lamb No. 5 (Table 6.16; Figure 6.39).

Campylobacters were recovered from the faeces of both lambs from the 2nd day PI onwards and at necropsy were present in all intestinal segments and showed the same pattern as previous lambs (Tables 6.17 and 6.18; Figures 6.40 and 6.41).

Blood cultures were negative. C. jejuni was isolated from the MLN, liver, spleen and lung of lamb No. 4 and from the MLN of lamb No. 5. E. coli was isolated from the MLN, livers, spleens and lungs

of both lambs. Streptococcus spp. was isolated from the MLN, livers, and lungs of both animals.

## 2.5 Comparison of C. jejuni biotype 1 strains colonization of gastro-intestinal segments of all lambs

The differences in campylobacter counts were analysed in two ways, firstly to assess the changes that appear to take place with age and secondly to see if the two C. jejuni strains behaved comparably. Erratic differences were found between lambs at different segments of the small intestine (Table 6.19) and were attributed to the age at which lambs were killed over the range 3 to 30 days PI, such that there was a significant negative regression of the mean logarithmic number of campylobacters on age ( $F_{(1,23)} = 9.86, p < 0.01$ ). The differences were not due to the strains; logarithmic counts of strain 476/82-D523-2 in lamb No. 1 (4th day PI) were comparable with the counts of strain 1744/81-B2464-2 in lambs No. 4 (3rd day PI) and 5 (7th day PI) ( $t_{(8)} = 2.105, p > 0.05$  and  $t_{(8)} = 2.574, p > 0.02$  respectively).

A similar relationship was not apparent between age and the mean counts from Co and Ca ( $F_{(1,8)} = 0.08, p > 0.05$ ). The Ab mean counts were similar to the mean counts of site 1 ( $t_{(4)} = 1.011, p > 0.10$ ).

## 3) Pathological studies

### 3.1 Gross findings

No gross abnormalities were found in any lamb. The stomachs contained clotted milk and the small intestine normal chyme. In all lambs the contents of the large intestine and faeces had a mucoid consistency. The mucosal surfaces did not show any alteration. The MLN, livers, spleens and lungs were apparently normal.

### 3.2 Histopathology

### 3.2.1 Lamb No. 4 (3rd day PI)

Intestinal changes were confined to site 5. Small aggregates of neutrophils were seen at the margins of Peyer's patch nodules, and a few of these cells with macrophages and eosinophils were detected in some villus cores. Neutrophils could also be observed apparently traversing the epithelium in the areas lined by "M" cells, sometimes forming a slight exudate at the luminal surface.

Tiny subepithelial foci of mononuclear cells and a few neutrophils were seen in the Ca but not in the Co.

### 3.2.2 Lamb No. 1 (4th day PI)

No alterations were found in sites 1 to 4. In site 5 incipient crypt abscesses (Figure 6.43) and more advanced lesions in which epithelial crypt cells were damaged with luminal pus were seen (Figure 6.44). However, Peyer's patches were normally developed (Figure 6.43) when compared with a control SPF lamb of 7 days of life (Figure 6.42). Purulent exudation was also found in the lumen of the ileum (Figure 6.45). Villi around Peyer's patches were short and contained granulocytes (mostly eosinophils) in some villus cores. In the submucosa peripheral to lymphoid follicles light neutrophil infiltrates were detected; these cells were also seen passing through the "M"-cell zone.

### 3.2.3 Lamb No. 5 (7th day PI)

No alterations were detected in sites 1 and 2. In sites 3 and 4 there were few neutrophils in the lamina propria between the crypts. In site 5 the lower lamina propria showed many eosinophils and a few neutrophils in tiny aggregates; some incipient crypt abscesses were found (Figure 6.46). Neutrophils were present at the peripheries of

the Peyer's patch nodules and could be seen in local capillaries. Occasionally on the luminal surface of site 5 clusters of short rod-shaped bacteria were seen near the surface of many villi distributed in a focal and irregular manner. Some of these villi contained few macrophages but were not stunted or altered in any way.

In Ca aggregates of mononuclear cells and a few neutrophils were seen in the lamina propria together with a few solitary crypt abscesses (Figures 6.47 and 6.48), changes which suggested a mild typhlitis.

#### 3.2.4 Lamb No. 2 (19th day PI)

Small intestinal sites were normal except site 2, where the villi tended to be "leaf-shaped" and some were infiltrated with mononuclear cells. Apart from the presence of small aggregates of macrophages and eosinophils, all large bowel sites were normal.

#### 3.2.5 Lamb No. 3 (30th day PI)

No significant pathological changes were seen at any site.

#### 3.2.6 Young's silver stains

Campylobacters and other rod-shaped bacteria were occasionally encountered, generally in small groups, in the lumen of the Ca and Co of all lambs (Figure 6.49). Often campylobacters were included into an amorphous mucus-like material which was very faintly stained and barely visible. In both sites, but more frequently in the Ca, isolated or small groups of campylobacters (Figures 6.50 and 6.51) were seen in the lumen of crypts. Campylobacters were not found either intracellularly or attached to epithelial cells. In contrast campylobacters were not found in the lumen or crypts of the small intestine.

### 3.3 Ultrastructural studies



TEM examinations of site 5 and Ca of lamb No. 5 corroborated the histopathological findings. Scattered crypt abscesses were found in both sites. Few neutrophils, lymphocytes, damaged epithelial cells and erythrocytes were occasionally found in the lumen. Campylobacters were found in small groups in the lumen of some crypts of the Ca, consistently surrounded by a mucus-like material as has been previously described in gnotobiotic calves (Figure 6.29).

No alterations were found by SEM examinations of site 5 of lambs No. 2 and 3. The apex or domes of Peyer's aggregated lymphoid tissues were well developed and visible from the surface. No campylobacters or other bacteria were detected on the surface of epithelial or "M" cells. All villi were morphologically normal.

SEM examination of the Co of lambs No. 2 and 3 showed a normal morphology throughout. This technique allowed the visualization of mucus droplets as they were extruded from goblet cells mouths and the formation of mucus particles and layers (Figure 6.52). No bacteria was found in lamb No. 2 but rods were observed included in mucus particles of lamb No. 3 (Figure 6.53). Adherent bacteria were not found in any case and only occasionally some epithelial cells undergoing necrobiosis were found (Figure 6.53).

SEM studies of the Ca of lambs No. 2 and 3 showed the presence of a few lymphocytes and epithelial cells on the luminal surface (Figure 6.54). Some epithelial cells undergoing necrobiosis bulged from the surface were distinguished by their separated microvilli (Figure 6.55). In most areas no bacteria were seen on the luminal surface (Figures 6.54 and 6.55) but in a small area of the Ca of lamb No. 3 campylobacters and other bacteria were seen on the surface or included in mucus

particles (Figure 6.56).

#### 4) Serological examinations

All agglutination tests were carried out using only the antigen of strain C. jejuni biotype 1 1744/81-B2464-2 because autoagglutination of antigen C. jejuni biotype 1 476/82-D523-2 prevented its use. Agglutinating C. jejuni antibody was detected in lambs No. 2 and 3 at titre of 1/80 in both animals. Sera of lambs No. 1, 4 and 5 did not agglutinate (less than 1/5).

Agglutination tests were also performed with C. coli antigen strain 569/82-D531-32 and the sera of all five lambs did not agglutinate (less than 1/5).

## II) Campylobacter coli infections

### Materials and Methods

Animals: three gnotobiotic lambs identified as follows: No. 6 (Black-face) and No. 7 and 8 (Greyface). Lamb No. 6 was maintained under gnotobiotic conditions in isolator G and lambs No. 7 and 8 were placed together into isolator H (Table 6.1).

Campylobacter oral inoculations: the inoculum was prepared, enumerated and orally administered as previously stated in Chapter 2. Lamb No. 6 was inoculated within the first 24 hours of life and lambs No. 7 and 8 at the 2nd day of life. The three lambs were infected with strain C. coli 569/82-D531-32 isolated from a diarrhoeic calf from A.D.R.A. outbreak D531, subgroup C1 (Table 4.23). Lamb No. 6 was inoculated on the 3rd of July 1982 with  $4.5 \times 10^8$  campylobacters ( $\log_{10} = 8.65$ ) and lambs No. 7 and 8 on the 29th of May 1983 with  $1.03 \times 10^9$  campylobacters

( $\log_{10} = 9.01$ ).

Necropsy: was carried out as previously described (Chapter 2) except that Re of lamb No. 6 was not sampled. Lamb No. 6 was killed at the 3rd day PI, lamb No. 7 at the 15th day PI and lamb No. 8 at the 26th day PI.

Bacteriological techniques: all campylobacter cultures were incubated at  $43^{\circ}\text{C}-550\text{H}_2$  for 20 hours. In lamb No. 6 faeces were cultivated daily for campylobacters on CBA plates but enumeration was not performed. In lambs No. 7 and 8 Miles and Misra's counts of faeces were carried out onto SA.

Campylobacter enumeration of gastrointestinal segments was performed in lamb No. 6 on BABA and in lambs No. 7 and 8 on SA.

Histological examinations: were carried out as described in Chapter 2 except that Ab and Re were not sampled in lamb No. 6. Young's stains were examined in sites 1, 2, 3, 4, 5, Ca and Co of lamb No. 6 and site 5, Ca, Co and Re of lambs No. 7 and 8.

Electron microscopy examinations: TEM was performed at Ca of lamb No. 7. SEM was carried out at site 5 of the same lamb.

Serology: plate agglutination tests were performed as previously described (Chapter 2) with antigens C. coli 569/82-D531-32 and C. jejuni biotype 1 1744/81-B2464-2.

## Results

### 1) Clinical observations

None of the lambs showed any apparent sign of illness or depression throughout the experiments. Milk intake was recorded only in lambs No. 7 and 8 up to the first feed at 11 days of life. Lambs No. 7 and 8 were respectively fed with 1.53 and 1.3 litres less than the standard

normal schedule, but these differences were found not significant when compared by Student's *t* test (Table 6.10). This milk consumption decrease occurred on days 4 to 8 (Appendix No. 3).

Faeces of lamb No. 6 were soft and contained mucus at the 2nd and 3rd day PI. Lambs No. 7 and 8 had soft mucoid faeces from the 3rd day PI up to the end of the experiments.

## 2) Bacteriological examinations

### 2.1 Lamb No. 6

This animal was maintained free from contaminants during all experiments and C. coli was the only microorganism isolated. Campylobacters were isolated from faeces on the 1st, 2nd and 3rd day PI. The number of campylobacters in the large intestine was  $10^3$  to  $10^5$  times greater than in the small intestine and Ab. Approximately  $10^3$  to  $10^4$  campylobacters per gram were enumerated in Ab, site 2, 3 and 4;  $10^5$  in sites 1 and 5 and  $10^9$  in Ca and Co (Table 6.20; Figure 6.57).

C. coli was isolated from blood cultures; it was not only isolated from both BSA bottles but also from a direct culture on a CBA plate. Campylobacters were also isolated from the MLN and spleen but not from liver or lung.

### 2.2 Lambs No. 7 and 8

Both animals were maintained free from bacteria up to the 2nd day of life (0 day PI). From the 1st day PI onwards E. coli was isolated from faeces of the two lambs. E. coli reached high levels in faeces from the 1st day PI onwards and was excreted by both lambs between  $10^9$  to  $10^{11}$  bacteria/g throughout the experimental period (Table 6.21; Figure 6.62). At post-mortem all gastrointestinal segments of both lambs had been successfully colonized by E. coli; the highest levels

were found in sites 4, 5, Co, Ca and Re ( $10^7 - 10^{10}$  bacteria/g) and the lowest in Ab and sites 1, 2 and 3 ( $10^5 - 10^7$  bacteria/g) (Table 6.22B; Figure 6.60). In the older lamb (No. 8) less numbers of E. coli were enumerated in all gastrointestinal segments except Re, but the difference of the mean logarithmic counts of all these segments excluding Re ( $t_{(14)} = 1.165$ ,  $p > 0.10$ ) or the small intestinal sites ( $t_{(8)} = 1.632$ ,  $p > 0.10$ ) was not significant. In Re an inverse relationship was found and approximately  $10^2$  more E. coli per gram were enumerated in lamb No. 8 than in No. 7. In lamb No. 8 from the 19th day PI onwards a yeast was isolated from SLM and Sabouraud plates; no attempt was made to enumerate the yeast in gastrointestinal segments.

C. coli faecal enumeration was performed from the 3rd day PI onwards. In lamb No. 8 a high level of campylobacters was detected from the first enumeration of faeces and was maintained throughout the experiment between  $10^9$  to  $10^{10}$  bacteria/g; in lamb No. 7 the number of campylobacters in faeces gradually increased from  $10^5$  up to  $10^{10}$  bacteria/g between the 3rd and 6th day PI and later was maintained at that level (Table 6.21; Figure 6.61). In both lambs the number of campylobacters in the large intestine was  $10^4$  to  $10^7$  times greater than in the small intestine. Approximately  $10^2$  to  $10^4$  campylobacters per gram were enumerated in the small intestine and  $10^8$  to  $10^9$  in the large bowel (Table 6.22A; Figures 6.58 and 6.59). The values obtained in lamb No. 8 correspond to colony counts of only one SA plate because in the duplicate microaerophilic jar growth was accidentally inhibited. In Re of lamb No. 7  $\log_{10} 1.67$  fewer campylobacters were found than in Co of the same animal, a difference that was significant at  $p < 0.01$  (Table 6.22A;  $t = 111.333$ ;  $p < 0.01$ ). On

the other hand Re of lamb No. 8 was similarly colonized as Co and Ca of the same animal (Figure 6.59).

Blood cultures were negative. C. coli was isolated from MLN, spleen and liver of lamb No. 7 and from MLN of lamb No. 8. E. coli was isolated from MLN, spleen, liver and lung of lamb No. 7 and from MLN and liver of lamb No. 8.

### 2.3 Comparison of C. coli colonization of gastrointestinal segments of lambs

The significance of differences between lambs No. 6 and 7 are shown in Table 6.23. Observation of Figures 6.57, 6.58 and 6.59 indicates that the number of campylobacters had decreased in the small intestine of the lamb No. 8, killed at the 26th day PI, whereas in the large bowel it is maintained at high levels at this stage ( $10^8 - 10^9$  bacteria/g). Although in lamb No. 8 it is clear that the number of campylobacters considerably diminish in the small intestine as time PI goes on this difference was not found between lambs No. 6 and 7. In fact Ab and site 3 of lamb No. 7 contained significantly more campylobacters per gram ( $p < 0.01$ ) than the corresponding sites of lamb No. 6. Other differences were present irregularly and were of limited significance. Not unexpectedly, therefore, the regression on time of the small intestinal counts of the 3 lambs was not significant ( $F_{(1,13)} = 1.28$ ,  $p > 0.05$ ).

### 3) Pathological studies

#### 3.1 Gross findings

Neither anatomical nor pathological abnormalities were found in the enteric tract, MLN, liver, spleen or lung of any of the lambs. In the three lambs the contents of the large intestine had mucoid

faeces.

### 3.2 Histopathology

#### 3.2.1 Hematoxylin-eosin stains

Lamb No. 6 (3rd day PI): all gastrointestinal segments were virtually normal. A few subepithelial aggregates of mononuclear cells could be seen in the Ca.

Lamb No. 7 (15th day PI): small intestinal sites were morphologically normal at all sites; infiltrates of eosinophils became progressively more numerous distally. Similarly, there was no morphologically significant change in the mucosa of the large bowel except for occasional focal infiltrates of mononuclear cells, mainly lymphocytes, between the upper parts of the glands. Less frequently, diffuse infiltrates of mononuclear cells and eosinophils with very few neutrophils were found. The Ca had the most prominent infiltrates and although most crypts were normal, isolated incipient crypt abscesses were detected (Figures 6.63 and 6.64). In the Re a few small foci of neutrophils just under the surface of the epithelium were found.

Lamb No. 8 (26th day PI): no significant changes were detected in any small intestinal site. Moderate numbers of eosinophils and mononuclear cells were seen in the lower mucosa. In the large intestine the alterations were limited to a few solitary lymphoid aggregates in the Co and a diffuse infiltration of mononuclear cells in both Co and Ca.

3.2.2 Young's silver stains: occasionally a few campylobacters and rod-shaped bacteria were found in the luminal surface of Ca, Co and Re of the lambs and only rarely isolated campylobacters were found in few crypts of the Ca. In no case were intracellular or attached



campylobacters were found. In small intestinal sites of lamb No. 6 and site 5 of lambs No. 7 and 8 campylobacters were not found either in the lumen or in cytoplasm of cells.

### 3.3 Ultrastructural studies

TEM examination of the Ca of lamb No. 7 demonstrated a normal morphology throughout. Occasionally isolated campylobacter-like organisms were found in the crypts near to enterocyte microvilli, but not attached and consistently embedded in amorphous mucus-like material much as described in gnotobiotic calves.

SEM examinations of site 5 of lamb No. 7 disclosed a normal morphology throughout, except for the presence of occasional completely or partially fused villi (Figure 6.65). Campylobacters were not seen on the surface of the enterocytes of the villi or "M"-cell clones covering Peyer's lymphoid follicles (Figure 6.66).

### 4) Serological examinations

In agglutination tests with the inocular strain sera from lamb No. 6 did not agglutinate (less than 1/5) and lambs No. 7 and No. 8 had a titre of 1/40. Sera from the three lambs did not agglutinate C. jejuni biotype 1 antigen No. 1744/81-B2464-2.

## III) "Campylobacter hyointestinalis" infections

### Materials and Methods

Animals: three gnotobiotic lambs identified as follows: No. 9 and 11 (Blackface) and No. 10 (Greyface). All animals were maintained together under gnotobiotic conditions in isolator I (Table 6.1).

Campylobacter oral inoculations: the inoculum was prepared, enumerated

and orally administered as previously described (Chapter 2). Each 1-day-old lamb was given on the 28th of May 1983  $1.25 \times 10^9$  ( $\log_{10} = 9.10$ ) bacteria. The three lambs were infected with CHI type 1 strain 213/82-B293-19 isolated from a diarrhoeic calf from A.D.R.A. outbreak B293, subgroup A2 (Table 4.23).

Necropsy: was carried out as previously described (Chapter 2). Lamb No. 9 was killed at the 6th day PI, lamb No. 10 at the 13th day PI and lamb No. 11 at the 25th day PI.

Bacteriological techniques: previous experience had shown that strain CHI type 1 NCTC11562 consistently grew on RNBGT at  $37^\circ\text{C}$ -650H<sub>2</sub> and that bovine CHI strains had been isolated from RNBGT (Chapter 3, Section C) therefore it had been decided to use RNBGT as a selective medium.

Due to a heavy growth of contaminants on RNBGT plates in all lambs from the 7th day PI onwards SA medium was introduced to replace RNBGT. All campylobacter cultures were incubated at  $37^\circ\text{C}$ -650H<sub>2</sub> for 3 days. Enumeration of contaminants was performed on CBA plus 0.2 per cent (w/v) of Bacteriological agar (Oxoid L11) in order to prevent development of spreading colonies.

In lamb No. 9 1/100 diluted homogenates of sites 3, 5 and Ca, and site 1 and Co were respectively filtered through 0.65  $\mu\text{m}$  and 0.80  $\mu\text{m}$  Millipore membranes and filtered drops were cultivated on CBA and incubated at  $37^\circ\text{C}$ -650H<sub>2</sub> for 6 days.

Histological examinations: were carried out as described in Chapter 2 except that Ab of lamb No. 9 was not sampled. Young's stains were examined in sites 3, 5 and Co of lamb No. 9, sites 2, 3, 5, Co, Ca and Re of lamb No. 10 and site 5, Ca, Co and Re of lamb No. 11.

Serology: CHI plate agglutination tests were performed as previously

described (Chapter 2) with antigen CHI type 1 213/82-B293-19.

## Results

### 1) Clinical observations

In lamb No. 9 the faeces had normal consistency throughout the experiment. In lambs No. 10 and 11 the faeces were pasty and relatively firm up to the 9th day PI on which mucus consistency was noted; from the 10th day PI onwards the faeces of both lambs acquired a soft consistency.

None of the lambs showed any other sign of illness throughout the experiment. In lambs No. 10 and 11 milk intake was recorded up to 11 days of life, compared with the expected milk intake for the corresponding period and found normal (Appendix No. 3; Table 6.10).

### 2) Bacteriological examinations

E. coli was isolated from the three lambs by direct culture of faeces at the 1st day of life (0 day PI). In all lambs faecal counts were performed from the 2nd day PI onwards and E. coli was found throughout the experiment at levels of  $10^9$  to  $10^{11}$  bacteria/g (Table 6.25; Figure 6.72). In lamb No. 11 Bacillus spp. was isolated by direct culture of faeces at the 15th day PI reaching levels of  $10^7$  to  $10^8$  bacteria/g from the 18th day PI onwards (Table 6.25). At post-mortem E. coli was found in the three lambs at levels of  $10^5$  to  $10^7$  bacteria/g in Ab and sites 1, 2 and 3, rising to  $10^8$  to  $10^{10}$  in large intestinal segments (Table 6.28; Figures 6.69 and 6.70); no significant differences were found between the mean logarithmic counts of all gastrointestinal segments ( $F_{(2,25)} = 1.09$ ,  $p > 0.05$ ). In lamb No. 11 Bacillus spp. colonized the Ab and small intestinal sites at levels of  $10^4$  bacteria/g and the large bowel at  $10^7$  to  $10^8$  bacteria/g (Table 6.28;

Figure 6.70).

On RNBGT plates campylobacters were overgrown by E. coli. This problem was solved when after comparative counts with non-inhibitory media SA was found to be suitable for the growth of the inocular CHI strain. SA was not used at the post-mortem of lamb No. 9 and for this animal isolation was attempted by means of Millipore membranes. CBA cultures obtained from 0.80  $\mu$ m membranes were partially contaminated by E. coli and 0.65  $\mu$ m were free from contaminants but campylobacters could not be recovered from any plate after 6 days of incubation. Afterwards all gastrointestinal homogenates of lamb No. 9 were stored for 5 days at  $-70^{\circ}\text{C}$ , thawed and directly cultivated and enumerated on SA; campylobacters could not be recovered from any direct culture and Miles and Misra's counts were negative. In order to evaluate these results the survival of campylobacters in homogenates of lamb No. 11 were enumerated before and after consecutive storage at  $+5^{\circ}\text{C}$  for 6 days and at  $-70^{\circ}\text{C}$  for 5 days (Table 6.24). After refrigeration a decrease of less than 10 bacteria/g was registered whereas after freezing as much as  $10^3$  to  $10^4$  bacteria/g had died. These results indicate that campylobacters in intestinal segments of lamb No. 9 should not have been higher than  $10^4$  to  $10^5$  bacteria/g. Support for this conclusion comes from a faecal sample of lamb No. 9 taken at the 4th day PI which after storage for 3 days at  $+5^{\circ}\text{C}$  contained  $10^4$  campylobacters/g on SA (Table 6.25).

The CHI excretion levels of lambs No. 10 and 11 were significantly different ( $p < 0.001$ ) when compared by Student's t test ( $t_{(16)} = 5.139$ ); in lamb No. 10 yielded between  $10^4$  to  $10^6$  campylobacters/g whereas in lamb No. 11  $10^6$  to  $10^9$  campylobacters/g were excreted (Table 6.25;

Figure 6.71). The logarithmic counts of lamb No. 10 (13th day PI, Table 6.26; Figure 6.67) were higher than the counts of lamb No. 11 (25th day PI, Table 6.27; Figure 6.68) in all gastrointestinal sites except in Re. Specifically the mean difference in the small intestine counts was significant when analysed by Student's paired t test and assuming that values  $< 2.70$  of lamb No. 11 were equal to 2.70 ( $t_{(9)} = 4.918$ ,  $p < 0.001$ ). In contrast the mean difference in the counts of the other gastrointestinal segments (Ab, Co, Ca and Re) was not significant ( $t_{(7)} = 1.224$ ,  $p > 0.10$ ). In Re of lamb No. 11  $10^3$  more campylobacters/g were enumerated than in Re of lamb No. 10, a difference which was highly significant when compared by Student's t test ( $t_{(2)} = 22.692$ ,  $p < 0.001$ ).

Blood cultures were negative. E. coli was isolated from the lungs, livers, spleens and MLNs of the 3 lambs and from the blood cultures of lamb No. 9. CHI and Bacillus spp. were only isolated from the MLN of lamb No. 11.

### 3) Pathological studies

#### 3.1 Gross findings

No anatomopathological abnormalities were found in the enteric tract and organs of any lamb. Mucoid contents were found in the large bowel of lambs No. 10 and 11.

#### 3.2 Histopathology

##### 3.2.1 Hematoxylin-eosin stains

Lamb No. 9 (6th day PI): no pathological changes were observed in any gastrointestinal segment. All tissues were identical to an SPF control lamb of similar age.

Lamb No. 10 (13th day PI): Ab was normal. In site 2 villi were

oedematous, in some areas they adhered together surrounded by a faintly stained mucus-like material and lacteals were distended. Villi were long and slender in all small intestinal sites, except site 5 where they were leaf-shaped, and were clad in normal columnar epithelial cells. A few macrophages and eosinophils were seen in some villi from site 3 increasing progressively and being numerous in site 5.

Large bowel segments were morphologically normal except for a few small aggregates of mononuclear cells found in the lamina propria of Co and a few macrophages scattered through the rectal mucosa.

Lamb No. 11 (25th day PI): no alterations were found in Ab. In the small intestine villi were leaf-shaped and in some areas of site 5 dome-shaped. Some villi had macrophages and eosinophils in their cores but otherwise they showed no significant changes. Crypts were apparently short. Eosinophils were numerous in the lower mucosa of all sites. Goblet cells were numerous in site 5.

All lower bowel segments appeared normal except for a few small aggregates of mononuclear cells in the lamina propria.

3.2.2 Young's silver stains: many rod-shaped bacteria were found in the lumen of site 5 and large intestinal segments of all three lambs but campylobacters were not identified. In site 2 of lamb No. 10 some villi were adherent together surrounded by a faintly stained mucus-like material which did not contain campylobacters but only some occasional bacilli. No intracellular bacteria were found in any of the examined segments.

#### 4) Serological examinations

Sera of lambs No. 9 and 10 did not agglutinate the inocular antigen. Serum from lamb No. 11 had a titre of 1/80.

## Discussion

In contrast to cattle, in which descriptions of natural and experimental enteritis due to campylobacters have been published (vide supra, Section A), in sheep very little evidence exists of campylobacters as cause of enteric disease either naturally (Russell, 1955) or experimentally (Bryans and Shephard, 1961; Firehammer and Myers, 1981). Under the conditions of gnotobiotic experiments, lambs excreted and were colonized by C. jejuni and C. coli strains, but 2 out of 3 lambs infected with CHI excreted significantly fewer campylobacters and the available evidence suggests (lamb No. 9) that early colonization at the 6th day PI was poor. This fact is coincident with the absence of CHI in any of 151 sheep faeces examined (Table 4.7B) and may indicate that this Campylobacter spp. is not completely adapted to colonize the ovine intestinal tract. The C. jejuni biotype 1 strains of different origin were used in experimental infections with 5 lambs and statistical comparisons of gastrointestinal segments (Table 6.19) did not show any difference between the colonization of the strains.

Despite the colonization by C. jejuni or C. coli, clinical signs of diarrhoea were not detected in any of the inoculated lambs. Instead a very mild disease was produced characterised by soft mucoid faeces and minor pathological changes which in some animals resembled those found in gnotobiotic calves (this Chapter, Section A). The pathological changes were assessed by comparison with non-inoculated SPF lamb histological preparations of which were available at A.D.R.A. Institute. Mucoid faeces, often of stringy consistency, as observed in these experiments has not been found in other SPF or gnotobiotic lambs not inoculated with campylobacters (K.W. Angus, Personal Communication,



1984).

Campylobacters colonized more successfully the large than the small intestine and it was a constant finding for the three Campylobacter spp. studied that as time PI goes on the number of campylobacters decreases in the small intestine whereas it was maintained at high levels in Co and Ca ( $10^8$  to  $10^{10}$  bacteria/g). Nevertheless, only in C. jejuni and CHI was the decline of small intestinal counts significant. Colonization of Re varied in some animals ( $10^5$  to  $10^{10}$  bacteria/g). When the three Campylobacter spp. were compared the differences in the mean total colonization of small intestinal sites of all lambs were not significant ( $F_{(2,48)} = 2.38, p < 0.05 > 0.01$ ). A comparison by Student's paired t test of the counts of the small intestine of lambs No. 2 (C. jejuni, 19th day PI) and 10 (CHI, 13th day PI) confirmed this finding, although the level of probability approached significance ( $t_{(9)} = 3.238, p < 0.05 > 0.01$ ). Earlier findings revealed a negative relationship between the number of campylobacters in the small intestine and age period. If colonization of C. jejuni and CHI had been similar the expected counts in the lamb killed at the 13th day PI would be higher than that of the animals killed at the 19th day PI. In fact, the opposite occurred, a finding that together with a significantly reduced CHI excretion rate (vide supra) support the hypothesis that CHI has a reduced ability to colonize.

In lambs inoculated with C. jejuni strains mild lesions characterised by the presence of crypt abscesses of different severity and inflammatory infiltrate in the adjacent lamina propria were found in site 5 of 3 lambs killed between the 3rd and 7th day PI and in Ca of the lamb killed at 7th day PI. Two other lambs killed at the 19th and 30th

day PI did not show any pathological alteration or any sign of proliferative enteritis. This clearly demonstrates that pathological changes mainly occur at the beginning of the infection and are of short duration. Similar incipient crypt abscesses were found in the Ca of a lamb inoculated with C. coli and killed at the 15th day PI but not in the animals killed at the 3rd and 26th day PI.

Campylobacters were not found intracellularly in any silver or EM preparations, but they were occasionally found in the lumen of crypts often in close proximity with the epithelial cells and consistently included in a mucus-like material. This suggests that, as previously described in calves, the most common mechanism of pathogenicity is probably the release of cytotoxic factors and also that campylobacters behave as mucosa-associated microorganisms (vide supra). Nevertheless, bacteremia did occur in lamb No. 6 (C. coli) and the isolation of campylobacters by direct blood culture indicates that at the 3rd day PI a high number of microorganisms had invaded the blood stream. Interestingly, no apparent clinical signs of disease or depression were evident when the lamb was taken from the isolator although rectal temperature and milk intake were not recorded.

Campylobacters were often isolated from the MLN of inoculated lambs.

In lambs inoculated with CHI different lesions were found in site 5 of lambs killed at the 13th and 25th day PI, and mainly consisted in shorter and dome-shaped villi respectively. No lesions were found in the lamb killed at the 6th day PI and this coincides with a poor colonization and excretion of CHI, although exact information of colonization could not be obtained in this animal. In none of the CHI lambs could be found lesions of proliferative enteritis or

intracytoplasmic campylobacters, although the experiment was maintained for 25 days in lamb No. 11 in which a relatively high excretion rate was recorded from the 4th day PI. Only in lamb No. 11 could campylobacters be isolated from MLN suggesting the less invasive ability of CHI compared with C. jejuni or C. coli. Crypt abscesses were not found in any of the 3 lambs inoculated with CHI indicating that this bacterium was not able to produce in gnotobiotic lambs the same inflammatory response and cytotoxic activity as the other Campylobacter spp. Al-Mashat and Taylor (1981) described in calves inoculated with CHI ("C. fecalis") abomasitis and in ileum prominent lymphoid tissue, stunting of the villi and dilated lacteals. In neither of the 2 lambs examined was abomasitis detected; the Peyer's lymphoid tissue was well developed but not different from control SPF lambs; stunted villi were evident in site 5 of lambs 10 and 11 and dilated lacteals in site 2 of lamb 10 in which oedema and adherence of villi by a mucus-like material was seen. These findings differ from the lesions described in conventional calves and this may be because lambs are not a susceptible host (Chapter 4, Section A).

The lesions in lambs were mainly localized in the ileum rather than in the large intestine as in calves. This characteristic makes the lesions of these gnotobiotic lambs more similar to those described in infected conventional calves (Al-Mashat and Taylor, 1980b and 1981) than those previously cited in gnotobiotic calves. The difference could be one of animal species or, that unlike the gnotobiotic calves used in this study most of the gnotobiotic lambs used here were contaminated with other bacteria either before inoculation or in the early stages of infection. The interference of colonization by normal

flora has been demonstrated in chickens and mice (Soerjadi, Snoeyembos and Weinack, 1982 and 1984; Andreumont, Leonard, Goldstein, Pean, Pequet and Tancrede, 1983) so probably other bacteria play an important role in the development of campylobacter lesions. Only lamb No. 6 (C. coli) was free from contaminants and interestingly no pathological changes were detected in the small intestine but minor alterations were found in the large bowel. The lack of non-contaminated campylobacter infected gnotobiotic lambs prevents further conclusions being made.

S. epidermidis colonization of these lambs differed notably from that described in gnotobiotic calves because this strain readily colonized the gastrointestinal tracts (Figure 6.37) and was excreted at high levels ( $10^8 - 10^{10}$  bacteria/g, Tables 6.12 and 6.14). These results agree with the described colonization by S. epidermidis of campylobacter inoculated gnotobiotic dogs (Prescott, Barker, Manninen and Miniats, 1981) and piglets (Kashiwazaki, Namioka and Yabiki, 1971).

Contaminants were detected in 10 out of the 11 lambs examined and in all the animals these bacteria were isolated from the MLN, in most (9 lambs) from other organs, and in lamb No. 9 E. coli was isolated from blood. Bacillus spp., Streptococcus spp., E. coli and S. epidermidis were isolated from MLN indicating that gnotobiotic lambs are highly susceptible to the spread of infection from gut lumen to the drainage lymph nodes and sometimes beyond by a variety of bacteria. Only one E. coli strain isolated from lamb No. 5 and in close association with the mucosa was serologically tested and found to be negative for K99<sup>+</sup> antigen (P. Wooding, Personal

Communication, 1984). *Campylobacters* behave similarly, jejuni/coli GC was re-isolated from the MLN of 7 out of 8 inoculated lambs and CHI from 1 out of 2 animals. The ability to invade may be due to the conditions of the experiment itself - highly susceptible animals and a large infective dose - rather than that the *campylobacter* strains tested were necessarily invasive under natural conditions.

Serum from two C. jejuni inoculated lambs, killed at the 19th and 30th day PI, agglutinated C. jejuni heterologous antigen whereas serum from the other 3 lambs inoculated at earlier dates did not have agglutinating antibodies against their inocular or heterologous strain. These results indicate that both C. jejuni biotype 1 strains used share a common superficial antigen besides their similar Penner's antigens (Table 4.23) and support the view that previous serological tests done on calves (Chapter 6, Section A) are valid. Serum from the lambs inoculated with C. coli and killed at the 15th and 26th day PI agglutinated the inocular C. coli antigen but not C. jejuni showing a specific serological response. Only lamb No. 11 killed at 25th day PI, produced agglutinating antibodies against the inocular CHI type 1 strain. In all cases the serological response occurred after 15 days of antigen exposure and the titres recorded were low either for C. jejuni and CHI (1/80) or C. coli (1/40). These results are in agreement with serological titres obtained in Campylobacter spp. infections in gnotobiotic calves (this Chapter, Section A) and piglets (Andress, Barnum and Thomson, 1968; Kashiwazaki, Namioka and Yabiki, 1971; McCartney, Lawson and Rowland, 1984) but differ from the results obtained with C. jejuni and CHI experimental infections in conventional calves (Al-Mashat and Taylor, 1980b and 1981) and pigs (Olubunmi and

Taylor, 1982) in which higher titres of 1/640 were detected between the 10th and 20th day PI. The possible reasons of this poor immunological response have been previously discussed.

After the experimental work re-isolated strains from lambs No. 1, 4 and 5 were examined and in all of them both types "M" and "N" colonies were found. These results clearly indicate that motile/non-motile variation is probably a common phenomenon in recently isolated strains and in many cases undetected when the culture medium is not adequate to demonstrate the different colony morphology. Similarly, as described in gnotobiotic calves type "N" colonies were more numerous in all gastrointestinal segments of lambs No. 2 and 3 except Re of lamb No. 3 and different levels of both colony types were also found in faeces. These two colonial types could not be related with any particular characteristic of the infection, it might be useful to perform experiments with separated clones of both types to assess if they behave differently.

CHAPTER 7FINAL DISCUSSION AND CONCLUSIONS

The primary aim of this study was an attempt to assess whether Campylobacter spp. were involved as a cause of enteric disease either in cattle or in sheep. Surveys were carried out to investigate the prevalence of campylobacters in diarrhoeic and non-diarrhoeic animals, to investigate their relationship with other enteropathogens and to obtain information on the epidemiology of such infections. These campylobacter strains were classified and compared with standard and other strains which have been previously described as agents of enteric disease or commensal bacteria of the gastroenteric tracts of ruminants, other animals and human beings. The most common Campylobacter spp. isolated from outbreaks of neonatal calf diarrhoea were used to infect young gnotobiotic calves and lambs to study whether campylobacters are able to produce enteritis per se.

Enteric bacteria may be present either as commensals or as pathogens, in this simplistic scheme the former are commonly supposed to populate the alimentary tract for long periods of time and are not associated with disease. Strains of particular species of commensal bacteria however show pathogenic properties not exhibited by all members of the species (eg. pathogenic E. coli) and thus may not be readily recognized in the laboratory. The campylobacters form a heterogenous group of bacteria in which differences in pathogenicity may exist between species or members of the same species: initial examination attempted to separate the individual types of campylobacters and to examine possible associations between their recovery and clinical disease. It is impossible at this stage to exclude the possibility that individual strains may not behave typically in terms of their pathogenicity.



Three groups of microaerophilic campylobacters were detected in the intestinal tract of ruminants, each comprising two related species: 1) jejuni/coli GC (C. jejuni and C. coli), 2) fetus GC (CHI and C. fetus) and 3) "atypical" fetus GC ("C. fecalis" and C. sputorum subsp. bubulus). The species within a group are closely related and within all 3 groups intermediate species have been found although these groups are clearly distinct. These groups of campylobacters are not only morphologically and biochemically different but also have different microaerophilic requirements which may indicate that they occupy different ecological niches in the intestine (Chapters 3 and 5).

C. jejuni and C. coli were both found either in sheep or cattle but other Campylobacter spp. differed in this respect. In cattle fetus GC was found, CHI being the most common species, whereas in sheep "atypical" fetus GC was detected with "C. fecalis" as the more frequent isolate. The bacteriological procedures were comparable so the results suggest that the campylobacters of these ruminants and their epidemiology differ. Despite the examination of 151 sheep faeces cultivated on suitable media, no recovery of CHI was made from sheep suggesting that these Campylobacter sp. either do not colonize at all or are rarely found (Chapter 4, Section A). These results are in agreement with the relatively poor colonization and low faecal excretion of 2 gnotobiotic lambs inoculated with CHI (Chapter 6, Section B).

Campylobacters were recovered from 45.6 per cent and 27.8 per cent of all cattle and sheep faeces examined respectively. As the bacteriological procedures were not optimal for the detection of some Campylobacter spp. and it has been demonstrated that natural campylobacter excretion can occur intermittently (Robinson, 1982) it seems

likely that if the animals had been examined repeatedly most would have been found infected at some stage. An example is the Easter Bush Flock in which the cumulative infection rate of 14 lambs increased from 7.1 to 78.6 per cent and different Campylobacter spp. were excreted for short periods by the same animal (Chapter 5, Section A). Other investigators found that the same calf yielded up to 4 serotypes of C. jejuni (García, Lior, Stewart, Ruckerbauer and Skljarevski, 1983 and Poster Communication, Second Workshop on Campylobacter Infections). It can then be concluded that campylobacters irregularly constitute part of the faecal flora of most ruminants, probably mainly located near the mucosal surfaces of the large intestine, where the oxygen tension and mucus secretion provide an appropriate environment for these microorganisms. The fact that campylobacters are commonly recovered from ruminants could be due to their ability to persist in the large intestine for relatively long periods of time. Once excreted in the faeces their persistence will result in opportunity for widespread dissemination and the infection of subsequent generations of animals.

In developed countries there is no doubt that jejuni/coli GC are aetiological agents of diarrhoeic disease in human beings because patients with diarrhoea more frequently excrete more campylobacters than healthy persons and illness was produced in volunteers who ingested cultures (Blaser and Reller, 1981). In this work C. jejuni, C. coli or other Campylobacter spp. were isolated from cattle and sheep but no correlation could be found between the excretion of campylobacters and diarrhoea (Chapter 4) and diarrhoeic disease was not produced when gnotobiotic calves and lambs were inoculated with C. jejuni, C. coli

or CHI isolates (Chapter 6). Similarly, the role of campylobacters as a human pathogen in developing countries has not been well established in all cases. For instance, in South Africa jejuni/coli GC was recovered as the sole bacterial pathogen from 31 per cent of 0 to 8-month-old children with acute gastroenteritis and from 5 per cent of asymptomatic children but in children of 8 to 24 months of age the respective recovery rates were 38 and 40 per cent, suggesting that the organism may be only pathogenic in very young children (Bokkenheuser, Richardson, Bryner, Roux, Schutte, Koornhof, Freiman and Hartman, 1979). In Bangladesh jejuni/coli GC were isolated from 38.8 per cent of healthy village 1-year-old children but nevertheless more infected children (48 per cent) had a history of recent diarrhoeal illness than did a group of matched controls (20 per cent), suggesting that campylobacter infection is common but generally related with subclinical disease (Blaser, Glass, Huq, Stoll, Kibriya and Alim, 1980). The fact that a mild disease was produced in gnotobiotic ruminants indicates that campylobacters are not merely commensal bacteria of the enteric tract but are able to produce minor pathological alterations and a few clinical signs which consist mainly of soft mucoid faeces occasionally containing blood. Conventional calves, inoculated with C. jejuni and C. fetus had also fever and diarrhoea with excess clear mucus and may contain blood (Al-Mashat and Taylor, 1980a and 1983b) and animals inoculated with CHI may have blood and mucus but the faecal consistency remains firm (Al-Mashat and Taylor, 1981). Other investigators found that C. jejuni inoculated conventional calves and lambs did not develop diarrhoea when not affected by other enteric disorders but frank or occult blood was detected in all animals (Firehammer and

Myers, 1981; Roberts, 1983). All these descriptions indicate that the mild clinical signs of these infections are generally undetected and could explain the poor correlation between campylobacter infections of ruminants and clinical enteric disease. Besides there are more serious diseases which produce severe diarrhoea, as for instance rotavirus, so that the detection of soft mucoid faeces or other mild clinical signs in young ruminants are not relevant to the practitioner and may be easily obscured by the other infections. Interestingly, when sheep were carefully observed by the author for minor alteration of faeces C. jejuni but not "C. fecalis" was increasingly excreted ( $p < 0.05$ ) by animals with mucoid soft faeces (Chapter 4, Section A, Slaughterhouse survey). This clinical examination was not made in other surveys, not only because the author had not taken the samples but also because in young milk-fed ruminants the faecal consistency is naturally soft and no available method to quantify mucus in faeces could be found.

As campylobacters might be more clearly identified as agents of calf diarrhoea without the interference of the most common enteropathogens, 370 calves from diarrhoeic herds which had been vaccinated against rotavirus and K99<sup>+</sup>EC were studied. These calves excreted more significantly campylobacters and cryptosporidia than animals from non-vaccinated herds but campylobacter excretion could not be related with presence of clinical diarrhoea whereas Cryptosporidium sp. was significantly more excreted than <sup>by</sup> control animals of other herds.

Field neonatal calf diarrhoea is a multiple agent disease in which specific agents cause or contribute to diarrhoea (Moon, McClurkin, Isaacson, Pohlenz, Skartvedt, Gillette and Baetz, 1978) and in this

work the relationship between the excretion of the examined enteropathogens was investigated (Chapter 4, Section B). It was found that 16.2 and 10.3 per cent more cryptosporidium infected calves respectively excreted fetus GC ( $p < 0.001$ ) and jejuni/coli GC ( $p < 0.01$ ) than animals without a detectable excretion of oocysts. These results indicate that calves parasited by Cryptosporidium sp. provide a better enteric environment for the multiplication of campylobacters and may suggest a synergistic action between the agents. Cryptosporidiosis alone is a cause of calf neonatal diarrhoea (Pohlenz, Moon, Cheville and Bemrick, 1978) so it would be of interest to pay attention to the relationship with campylobacter excretion in future epidemiological studies and also to include the parasite in combined infection studies. Piglets are susceptible to oral infection with Cryptosporidium sp., originally derived from diarrhoeic calves (Tzipori, McCartney, Lawson, Rowland and Campbell, 1981), but cryptosporidial infection did not influence the colonization patterns of C. sputorum subsp. mucosalis or promote the establishment of significantly higher numbers of campylobacters (McCartney, 1982). Despite the absence of effect on campylobacter colonization in piglets, investigation in calves with different Campylobacter spp. and Cryptosporidium sp. have not been made and may provide different results.

Interestingly, statistical analysis of data in Chapter 4, Section B showed that young dairy calves excreted significantly less ( $p < 0.001$ ) jejuni/coli GC than beef animals whereas the isolation rate of fetus GC and the other 4 agents examined was similar in both types of herds. This observation is new and suggests a different epidemiological behaviour of these two campylobacter groups, possibly brought about by management or feeding practices.

These investigations have been designed to study the possible role of campylobacters as agents of diarrhoea in young calves of less than 1 month of life, an age at which the disease is frequent and of economical importance. Al-Mashat and Taylor (1980b, 1981 and 1983b) were able to produce enteric disease in 4 to 6-month-old ruminanting calves with C. jejuni, C. fetus or CHI but specifically with the latter the disease was not produced in milk fed calves suggesting that old animals may be more susceptible than young. The reasons for a possible increased susceptibility of old animals may be due to a different microbial population of the intestine which could alter the gaseous environment and govern the location of the campylobacters in the gastrointestinal tract. Campylobacters as strict microaerophilic microorganisms in an anaerobic environment may be compelled to grow close to epithelial cells of the gut where theoretically the oxygen tension is greater. Campylobacters in close proximity to epithelial cells may be more pathogenic than organisms located in the lumen. Experiments in gnotobiotic ruminants (Chapter 6) showed that campylobacters are more likely to act by the local release of cytotoxic factors rather than the invasive ability of the bacteria. Further evidence may be deduced by a comparison of the location of the intestinal lesions in lambs and in calves, in the former where pre-infection contamination had occurred lesions were more common in the small intestine than in the germ-free campylobacter infected calves. Acquired immunity is likely in some instance to result in the termination of excretion and this is the normal pattern of human infection, such individuals are however susceptible to re-infection by different serotypes (Karmali, Kosoy, Newman, Tischler and Penner, 1981) and

similarly Roberts (1983) performing re-infection experiments with cattle and sheep found that resistance occurred to homologous but not heterologous Penner's serotype challenge. The diversity of Campylobacter spp. and serotypes can explain why although these micro-organisms are so common, complete protection is not achieved and old animals may be susceptible to re-infection with different serotypes.

The selective bacteriological media used in this thesis (PN) has notably improved the isolation of campylobacters from ruminant faeces controlling almost completely contaminant bacteria to such an extent that it is possible to enumerate campylobacters in gastrointestinal segments of conventional ruminants. This information is crucial to interpret whether the association of the lesions in conventional and gnotobiotic calves corresponds to the best colonized areas and the role of the normal flora in the development of the disease.

The use of selective media and only one microaerophilic atmosphere limited these studies and the isolation obtained may represent only part of a more complex campylobacter flora. It is therefore possible that further Campylobacter spp. exist but have not been detected; future investigations should include different culture media and isolation procedures.

It can be concluded that the 3 groups of campylobacters studied here comprise very heterogenous bacteria which are commonly found in the faecal flora of sheep and cattle and from which some of their species are able to produce a relatively mild self-limiting disease in young ruminants, characterised by minor pathological alterations which tend to resolve rapidly.



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## APPENDICES

## APPENDIX No. 1 Daily milk intake of gnotobiotic calves (in litres)

Days of life	Standard milk intake	Calf No. 1	Calf No. 2	Calf No. 3
0	2	2	1.95	2.6
1	2	2	2.1	2.2
2	2	2	2.1	2.1
3	2	2	2.1	2.2
4	2	2	2.1	2.3
5	2	2	2.1	2.2
6	2	2	2.1	2.4
7	2	2	2.1	2.4
8	2.5	0.9(*)	2.5	2.4
9	2.5	-	2.5	2.5
10	2.5	-	2.5	2.4
11	3	-	2.5	2.4
12	3	-	2.5	2.4
13	3	-	2.5	2.4
14	3	-	2.5	3
15	3.5	-	0.9(*)	3.6
16	3.5	-	-	3.6
17	3.5	-	-	3.6
18	3.5	-	-	3.6
19	3.5	-	-	3.6
20	3.5	-	-	3.6
21	3.5	-	-	3.6
22	3.5	-	-	3.6
23	3.5	-	-	1.2(*)

(\*) : last day of life includes only one feed



APPENDIX No. 2 A.D.R.A. standard milk intake schedule of gnotobiotic lambs (in litres)

Days of life	1st Feed	2nd Feed	3rd Feed	4th Feed
0	0.50-0.1 <sup>(*)</sup>	0.1-0.15 <sup>(*)</sup>	0.15-0.2 <sup>(*)</sup>	0.2 <sup>(*)</sup>
1	0.25	0.2	0.25	-
2	0.3	0.25	0.35	-
3	0.35	0.3	0.35	-
4	0.4	0.35	0.4	-
5	0.45	0.4	0.45	-
6	0.45	0.45	0.45	-
7	0.75	-	0.75	-
8	0.8	-	0.8	-
9	0.8	-	0.9	-
10	0.8	-	0.9	-
11	0.9	-	0.9	-
12	0.95	-	0.95	-
13	0.95	-	0.95	-
14	1	-	1	-
15	1	-	1	-
16	1	-	1	-
17	1	-	1	-
18	1	-	1	-
19	1	-	1	-
20	1	-	1	-
21	2	-	-	-

Continue until the end of experiment

(\*) : half strength milk

APPENDIX No. 3 Daily milk intake of gnotobiotic lambs ( in litres)

Days of life	Lamb No.1	Lamb No.4	Lamb No.5	Lamb No.7	Lamb No.8	Lamb No.10	Lamb No.11
0	0.525	0.65	0.65	0.45	0.5	-	-
1	0.75	0.7	0.7	0.7	0.7	0.7	0.7
2	0.9	0.9	0.9	0.85	0.85	0.85	0.85
3	1	1	1	1	1	1	1
4	0.4(*)	0.4(*)	1.15	1.1	1.1	1.15	1.15
5	-	-	1.3	1.13	1.2	1.35	1.3
6	-	-	1.35	1.14	1.2	1.4	1.4
7	-	-	1.6(*)	1.15	1.05	1.5	1.5
8	-	-	0.8(*)	1.15	1.3	1.5	1.5
9	-	-	-	1.7(*)	1.7	1.7	1.7
10	-	-	-	0.6(*)	0.6(*)	1.7	1.7
11	-	-	-	-	-	0.75(*)	0.75(*)

- : not available data or animal killed

(\*) : included only first feed